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Avaliação da toxicidade subcrônica de bebida energética associada ao álcool em ratos Wistar machos e fêmeas durante a puberdade

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A todas as mulheres que fizeram,
fazem e farão Ciência em meio
a um mundo tão turbulento.

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(Muriel Barbery)

RESUMO

O consumo de energéticos cresce entre os jovens e este consumo tem se tornado mais expressivo, inclusive quando associado ao álcool. Os principais constituintes desta bebida são cafeína e taurina, um estimulante e um depressor do sistema nervoso central, respectivamente. Este estudo teve como objetivo avaliar a toxicidade de bebida energética, seus constituintes cafeína e taurina, isolados e em associação ao álcool em ratos Wistar pré-púberes utilizando o protocolo descontínuo de administração repetida do tipo *binge drinking* e avaliando parâmetros: comportamentais, hematológicos, bioquímicos, imunológicos, oxidativos, histopatológicos e reprodutivos. Ratos Wistar machos e fêmeas (pré-púberes) foram tratados do 32º dia de vida por gavagem através do protocolo *binge drinking* (3 dias consecutivos por semana, com intervalo de 4 dias por semana, ao longo de 4 semanas – mimetizando o uso intermitente pelos jovens) com água destilada (10 mL/kg), energético (10 mL/kg), cafeína (3,2 mg/kg), taurina (40 mg/kg) e álcool (2 g/kg, 20%) de forma combinada ou isolada. A análise hematológica foi feita através da análise do sangue no Micros-60, as análises bioquímicas foram feitas através de marcadores renais e hepáticos no espectrofotômetro X BS-120. A histopatologia foi feita através da fixação em hematoxilina e eosina e posterior leitura em microscópio óptico (40x). As citocinas (TNF-α e IL-1β) foram avaliadas através do método de Elisa, os níveis de espécies reativas de oxigênio (ROS) através da intensidade de fluorescência ao redox DCFH, e os níveis de óxido nítrico pelo método de Griess. As avaliações comportamentais avaliaram a locomoção através do teste Open Field, ansiedade pelo teste Pluz Maze e memória atrvés do teste de reconhecimento de objetos. As avaliações reprodutivas foram feitas através do peso dos órgãos sexuais, análise do ciclo estral das ratas fêmeas, qualidade dos oócitos através da liberação dos complexos cúmulos oócitos (CCOs), contagem de espermatozoides e espermátides, avaliação morfológica de espermatozoides e nível de testosterona. Os resultados apresentaram aumento significativo de citocinas inflamatórias (TNF-α e IL-1β), principalmente nos grupos tratados com energéticos, álcool e álcool associado ao energético foram encontrados no sangue periférico dos animais. Assim como, um amento da presença de alterações histopatológicas no fígado e nos rins (vacuolização, infiltrado celular e degeneração hidrópica) sobretudo nos grupos tratados com álcool. Porém, as avaliações bioquímicas, hematológicas e reprodutivas não apresentaram diferença entre os grupos tratados. Nas avaliações comportamentais, o teste de locomoção (teste do campo aberto) nas fêmeas reforça a ação estimulante da cafeína e do energético nos cruzamentos externos. Nos machos, a cafeína e a taurina melhoraram o índice de reconhecimento de objetos no teste de memória de longa duração. Em conjunto, os dados mostram que o álcool isolado e associado ao energético causa prejuízo ao organismo, assim como o energético isoladamente. Porém, os constituintes dos energéticos (cafeína e taurina) mostraram-se protetivos mesmo em doses baixas.

Palavras-chave: energéticos, cafeína, taurina, álcool, ratos pré-púberes, *binge drinking*

ABSTRACT

The consumption of energy drinks is growing among young people, this consumption has become more expressive, even when associated with alcohol. The main constituents of this drink are caffeine and taurine, a stimulant and a depressant of the central nervous system, respectively. This study aims to evaluate the toxicity of energy drink, its constituent's caffeine and taurine, isolated and in association with alcohol in prepubertal Wistar rats using the discontinuous protocol of repeated administration of the binge drinking type and evaluating parameters: behavioral, hematological, biochemical, immunological, oxidative, histopathological and reproductive. Male and female Wistar rats (prepubertal) were treated from the 32nd day of life by gavage through the Binge Drinking protocol (3 consecutive days a week, with an interval of 4 days a week, over 4 weeks - mimicking the intermittent use by young people) with distilled water (10 mL/kg), energy drink (10 mL/kg), caffeine (3.2 mg/kg), taurine (40 mg/kg) and alcohol (2 g/kg, 20%) so combined or isolated. Hematological analysis was done through blood analysis on the Micros-60, biochemical analyzes were performed using kidney and liver markers on the X BS-120 spectrophotometer. Histopathology was performed by fixation in hematoxylin and eosin and subsequent reading under an optical microscope (40x). Cytokines (TNF-α and IL-1β) were evaluated by the Elisa method, the levels of reactive oxygen species (ROS) by the redox fluorescence intensity DCFH, and the nitric oxide levels by the Griess method. Behavioral assessments assessed locomotion through the Open Field test, anxiety through the Pluz Maze test and memory through the object recognition test. Reproductive evaluations were made through the weight of Organs sexual organs, analysis of the estrous cycle of female rats, oocyte quality through the release of cumulus oocyte complexes (CCOs), sperm and sperm counts, morphological evaluation of sperm and testosterone level. The results showed a significant increase in inflammatory cytokines (TNF-α and IL-1β), especially in the groups treated with energy drinks, alcohol and alcohol associated with energy drinks were found in the peripheral blood of the animals. As well as an increase in the presence of histopathological changes in the liver and kidneys (vacuolization, cellular infiltrate and hydropic degeneration) especially in groups treated with alcohol. However, biochemical, hematological and reproductive evaluations showed no difference between the treated groups. In behavioral assessments, the locomotion test (open field test) in females reinforces the stimulating action of caffeine and energy drink in external crossings. In males, caffeine and taurine improved the object recognition index in the long-term memory test. Together, the data show that alcohol alone and associated with energy drink causes harm to the body, as well as energy drink alone. However, the constituents of energy drinks (caffeine and taurine) proved to be protective even at low doses.

Keywords: energy drinks, caffeine, taurine, alcohol, pre-pubertal rats, binge drinking

Lista de abreviaturas e siglas

ANVISA	Agência Nacional de Vigilância Sanitária
AST	Aspartato Transaminase
ALT	Alanina Transaminase
BD	*Binge Drinking*
CAT	Catalase
CCL_4	Tetracloreto de Carbono
CCO	*Complexos Cumulus-oócito*
DCFH	Diclorofluoresceína
DNA	Ácido desoxirribonucleico
FA	Fosfatase Alcalina
FDA	Food and Drug Administration
FSH	Hormônio Folículo Estimulante
g	Grama
GABA	Neurotransmissor do tipo Ácido Gama-aminobutírico
GAD	Ácido Glutâmico Descarboxilase
GFAP	Proteína Ácida Fibrilar Glial
GLY	Glicina
GnRH	Hormônio liberador de gonadotrofina
GPX	Glutationa Peroxidase
GSH	Glutationa reduzida
IFN-α	Interferon α
IL	Interleucina
iNOS	Óxido Nítrico Sintase Induzível
kg	Kilograma
L	Litro
LH	Hormônio luteinizante
MDA	Malondialdeído
mL	Mililitro
mM	Milimolar
mg	Miligrama
NMDA	N-metil D aspartato
NO	Óxido Nítrico
PRL	Prolactina

ROS	Espécies Reativas de Oxigênio
RDC	Resolução da Diretoria Colegiada
STZ	Estreptozotocina
TLR	Receptor *Tool Like*

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Sumário

1 REVISÃO DA LITERATURA

1.1 Introdução

As bebidas conhecidas como energéticos foram lançadas nos anos 60 na Europa e na Ásia, porém tiveram o reconhecimento mundial ao atingir o mercado americano em 1997 com a marca Red Bull®. Tais bebidas são a base de cafeína e tem como intuito proporcionar um impulso de energia e aumentar o estado de alerta (PENNAY et al., 2012) além de evitar o sono e aumentar o estado de concentração (BALLISTRERI E CORRADI-WEBSTER, 2008). O consumo anual de bebidas energéticas em 2013 excedeu 5,8 bilhões de litros em cerca de 160 países (BAILEY et al., 2014). Com venda anual é de cerca de 50 bilhões de dólares, tem como projeção até 2021 atingir cifras de 60 bilhões de dólares (CURRAN e MARCZINSKI, 2017).

A maioria (2/3) dos consumidores são do gênero masculino, entre 13 e 35 anos. Nos Estados Unidos, as bebidas energéticas são o segundo suplemento alimentar mais regularmente utilizado por adultos jovens (30%) (SIMON et al., 2007). A indústria multibilionária do mercado dos energéticos usa estratégias agressivas e inovadoras de *marketing* para atingir adolescentes e jovens (HOWLAND et al., 2013), incluindo a promoção destes, em eventos esportivos e festivais de música (WOLK et al., 2012).

Os principais constituintes dos energéticos são cafeína, taurina, guaraná, açúcar, sódio e vitamina B6 (ISHAK et al., 2012). Além disso, são incluídos na composição de algumas marcas, glucuronolactona, ginseng, *Ginko biloba* e outros constituintes (HIGGINS et al., 2010). Apesar de possuírem alguns efeitos benéficos como melhora da memória, aumento da vigilância e humor, também são relatadas alterações cardiovasculares, neurológicas, psicológicas, gastrointestinais, metabólicas e renais (ALSUNNI, 2015). No Brasil, de acordo com a ANVISA, atribui-se o uso da expressão "bebida energética" ou "*energy drink*" ao produto que contém em sua composição inositol e/ou glucoronolactona e/ou taurina e/ou cafeína, podendo ser adicionado de vitaminas e minerais e, inclusive, de outros ingredientes, desde que estes não descaracterizem o produto. As bebidas energéticas vendidas no Brasil não podem conter quantidades superiores a 35 mg/100mL de cafeína e 400 mg/100 mL de taurina, definidas na resolução RDC ANVISA 273/2005 (SECRETARIA DE VIGILÂNCIA SANITÁRIA DO MINISTÉRIO DA SAÚDE, 2005).

Nos últimos anos, o número de casos nos serviços de emergência dos Estados Unidos relacionados às bebidas energéticas dobrou de 10.068 em 2007 para 20.783 em 2011(MATTSON, 2013). Em contrapartida, um estudo revelou que a maioria (64,3%) dos estudantes que fazem uso da substância não tem conhecimento dos seus possíveis efeitos colaterais (SCURI et al., 2018). Devido aos seus efeitos adversos, um potencial perigo a crianças e adolescentes têm sido atribuídos aos energéticos. Por isso, a Academia Americana de Pediatria elaborou algumas recomendações, entre elas: 1) cautela quanto aos possíveis riscos para crianças e adolescentes devido aos compostos estimulantes contidos na bebida; 2) maior suscetibilidade a uma grande exposição dos ingredientes ativos numa base por quilograma; 3) possível ingesta excessiva de carboidratos que pode causar sobrepeso (COM & COSMF, 2011).

Além do consumo recorrente de energéticos, por adolescentes e adultos jovens, torna-se cada vez mais frequente a associação com bebidas alcoólicas como a vodka (PENNAY et al, 2012). Em um estudo conduzido nos 16 países membros da União Europeia, o qual mais de 52.000 indivíduos foram entrevistados. Observou-se que 53% dos adolescentes fazem uso de bebidas energéticas associadas ao álcool (ZUCCONI et al., 2013). Acredita-se que o efeito estimulante da cafeína possa antagonizar os efeitos sedativos do álcool, possivelmente levando a um aumento do consumo de álcool e consequentemente, aumentando os efeitos tóxicos (HANN et al, 2012, HOWLAND et al, 2011, FERREIRA et al, 2004). Outros efeitos relacionados incluem o aumento do risco de acidentes e violência, a associação com outras drogas e piores desempenhos escolares (BERGER et al, 2013; TUCKER et al, 2016). Os indivíduos que ingerem energéticos acreditam que são mais capazes de executar comportamentos que exijam controle motor fino, apesar de estarem debilitados pelo álcool, mascarando os sintomas subjetivos da intoxicação alcoólica (CURRY et al, 2009; SNIPES et al, 2013). Pesquisas revelam ainda que a percepção de cefaleia, xerostomia e prejuízo da coordenação motora diminui quando os energéticos são consumidos em associação com o álcool em comparação ao álcool isoladamente (VERSTER et al, 2012). Considerando estes achados, em 2010, o FDA (*Food and Drug Administration*) determinou que a associação de cafeína e álcool não é segura (FDA, 2010).

1.2 Cafeína

Dentre os principais compostos encontrados nos energéticos e, de maior relevância, encontra-se a cafeína (1,3,7-trimetilxantina) o componente psicoativo primário. A cafeína é absorvida rápida e completamente pela via oral, tendo meia vida de aproximadamente 4,5 h (GLADE et al.; 2010 WOLK et al., 2012), exercendo seus efeitos psicoestimulantes tanto em humanos quanto em ratos (BOECK et al., 2009). Dentre as suas atividades pode-se destacar aumento no estado de alerta e redução do cansaço. Em contrapartida, ela pode estar relacionada ao aumento de ansiedade, pois indivíduos que consomem cafeína em grandes quantidades podem tornar-se ansiosos. (SMITH, 2002). Doses elevadas de cafeína podem causar palpitações, hipertensão, estimulação do sistema nervosos central, náusea, vômito, hipocalcemia, acidose metabólica, convulsões e, em casos raros, a morte (BREDA et al, 2014).

Estudos farmacológicos indicam que os efeitos da cafeína no sistema nervoso central são mediados particularmente pela ação antagonista de receptores de adenosina dos subtipos A1 e A2 (LORIST et al, 2003). Também tem sido demonstrado que a cafeína induz a liberação de dopamina no estriado e no córtex pré-frontal. A função da dopamina no estriado é importante tanto para estimular quanto ativar o mecanismo de recompensa de várias drogas, incluindo os efeitos hedônicos do álcool. O mesmo mecanismo pode mediar o aumento do desejo de beber álcool que é visto com o consumo junto aos energéticos (McKETIN, et al 2015). Os receptores de adenosina estão envolvidos na regulação da produção de espécies reativas de oxigênio (ROS), afetando a gênese e o impacto de radicais livres em neurônios e em outros sistemas biológicos (ABREU et al, 2011). As espécies reativas de oxigênio (ROS) estão envolvidas em dano tecidual através de uma variedade de insultos. Estas substâncias podem diretamente danificar proteínas, DNA, lipídeos e, portanto, afetar muitas funções celulares (NOSCHANG et al, 2009).

De acordo com a revisão de Martini e colaboradores (2016), o efeito do consumo de café em humanos, tanto em exposições crônicas quanto agudas, sobre diferentes parâmetros de estresse oxidativo geram dados conflitantes e inconsistentes quanto ao real efeito do café/cafeína no potencial oxidativo. Dentre estes, quanto a capacidade antioxidante total, atividade de enzimas antioxidantes superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx), dano lipídico e dano a proteínas.

Além dos reconhecidos efeitos estimulantes da cafeína, outros são relatados, por exemplo, efeitos pró-inflamatórios. Frau e colaboradores (2016) investigaram níveis de TNF-α (Fator de necrose tumoral α), IL-1β (Interleucina 1β) e nNOS (óxido nítrico sintase neuronal) nas estruturas do putâmen, caudado (CPu) e na substância nigra pars

compacta (SNc) de camundongos pré-púberes (28 dias) tratados com cafeína (10 mg/kg) ou MDMA (3,4-metilenodioximetanfetamina - 4 x 20 mg/kg), tanto isolados quanto em associação. Neste estudo foi demonstrado que a associação dos compostos aumentou os parâmetros neuro-inflamatórios e neurotóxicos avaliados. Entretanto, Machado-Filho e colaboradores (2014) avaliaram os efeitos da cafeína (10 e 20 mg/kg, diariamente por 2 semanas) em ratos Wistar machos com Parkinson induzido por 6-OHDA, observando diminuição de citocinas pró-inflamatórias como IL-1β e TNF-α encefálicas no estriado e na substância nigra, bem como aumento do conteúdo de dopamina no estriado. Corroborando com estes achados, referentes à ação anti-inflamatória da cafeína, outros autores, avaliaram o efeito desta (20 mg/kg) em modelo de Parkinson induzido por Paraquat e Maneb, onde foi possível demonstrar que a cafeína diminui os níveis de óxido nítrico (NO) na região nigroestriatal e ativa as micróglias (na substância nigra), conferindo neuroproteção contra a neurodegeneração dopaminérgica (YADAV et al., 2012).

Os efeitos da cafeína também é alvo de pesquisa na avaliação do sistema reprodutor. Alguns estudos demonstram que a cafeína atua em homens e mulheres diminuindo a fecundabilidade em ambos os sexos. Em casos em que o consumo excede 699 mg/dia, a taxa de fecundabilidade pode ser diminuída em 44%. (SADEU, 2010). Em filhotes machos de ratas que receberam 26 e 45 mg/kg de cafeína no período gestacional, foi observado perda da espermatogênese, redução da massa dos testículos, menor diâmetro dos túbulos seminíferos e redução do tamanho do epitélio do tubo germinativo Na prole oriunda do grupo de alta dose e foi demonstrado, ainda, diminuição na massa do epidídimo, próstata e vesícula seminal, e anormalidades na morfologia espermática, além de redução na quantidade de espermatozoides (DOROSTGHOAL et al., 2012). Além disso, quebras de DNA e aneuploidias espermáticas foram relatadas em um estudo transversal com homens entre 19 e 35 anos de idade que relataram uso de cafeína (ROBBINS et al., 1997).

ZHANG e colaboradores (2017), tratou camundongos Kunming de (6 a 8 semanas) com gonadotrofina sérica de égua prenhe (PMSG) e gonadotrofina coriónica humana (hCG), para provocar superovulação. Após a eutanásia dos animais, houve a liberação dos complexos cumulus dos oócitos (COCs). Os COCs de três camundongos tiveram sua maturação *in vitro* para tratamento com cafeína e após, foram incubados e suplementados com 5 mM cafeína durante 24 horas. As análises microscópicas

evidenciaram que a cafeína inibiu a separação de células do cumulus de oócitos (COCs), retardando a maturação.

1.3 Taurina

Outro componente amplamente encontrado nos energéticos é a taurina (ácido 2-aminoetanossulfónico), aminoácido mais abundante no sistema nervoso central (HUSSY et al., 2000), cujo impacto do uso de altas concentrações ainda não é bem conhecido (RATH et al, 2012, SEIFERT et al, 2011). Em mamíferos possui funções fisiológicas importantes como: antioxidante, osmorregulador, estabilizador de membrana e neurotransmissor. A taurina atua como neurotransmissor inibitório através dos receptores de GABA (ácido gama aminobutírico) e Gly (glicina), tendo efeito ansiolítico em camundongos e ratos (CHEN et al., 2004; KONG et al., 2006).

Alguns dos efeitos da taurina são atribuídos a um papel na função de memória através da modulação de receptores de N-metil-D-aspartato (NMDA). Porém nenhum estudo em humanos estabeleceu uma relação concreta entre taurina e melhora da memória (BICHLER et al, 2006). Também vem sendo estudado o efeito da taurina na excitabilidade neural através da alteração do sistema inibitório GABAérgico, incluindo elevação na expressão do ácido glutâmico descarboxilase (GAD), aumento dos níveis de GABA, e baixa regulação do receptor GABAA (CALABRÓ et al, 2012).

Sendo um antioxidante eficaz, a taurina identifica a presença de espécies reativas de oxigênio e estimula enzimas antioxidantes direta ou indiretamente (XU et al, 2015). Neste sentido, Patel e colaboradores (2016) avaliaram o efeito da taurina em diferentes doses (1,2; 2,4 e 3,6 mM/kg) no tratamento de ratos diabéticos induzidos por STZ (estreptozotocina), onde foi detectado aumento da atividade das enzimas SOD, CAT e GPx na medula espinhal, córtex, cerebelo e tronco cerebral, indicando seu papel protetor contra estresse oxidativo. Além disso, Abdel-Moneim e colaboradores (2015) comprovaram que a associação de taurina (100 mg/kg) e silimarina (100 mg/kg) (um flavonóide polifenólico antioxidante que protege contra lesões hepáticas provocadas por hepatotoxinas) foi capaz de proteger ratos com dano hepático induzido por tetracloreto de carbono (CCL_4) contra o estresse oxidativo. Neste modelo observou-se o aumento da atividade enzimática de SOD e GPx, diminuição da peroxidação lipídica, diminuição dos níveis de NOS, NO, e das enzimas hepáticas (AST: Aspartato Transaminase, ALT:

Alanina Transaminase, ALP: Fosfatase Alcalina), bilirrubina total e hidroxiprolina. Além disto, a taurina, isoladamente diminuiu os níveis de TNF-α no soro.

A Taurina (15 mM) foi estudada em um modelo *in vitro* de propriedades antioxidantes, e observou-se que atua como um eliminador de oxigênio reativo (radical peroxil e ánion superóxido) e de espécies reativas derivadas de nitrogênio (óxido nítrico e peroxinitrito) (OLIVEIRA et al 2010). Ainda de acordo com Jong et al (2012) a taurina serve como um regulador da síntese proteíca mitocondrial, aumentando a atividade da cadeia transportadora de elétrons e protegendo as mitocôndrias contra a geração excessiva de superóxido. Avaliando tais efeitos da administração de taurina (50, 250 e 500 mg/kg), sobre o sistema antioxidante, ratos Wistar foram tratados durante 60 dias, e foi realizada análise enzimática (GPx, GSH, GR) nos órgãos (rim, fígado, coração e estômago). Os resultados mostraram uma variação enzimática de órgão para órgão e, concluiu-se que o longo tempo de administração da taurina por via oral causou efeito nos níveis normais da atividade antioxidante de suas enzimas (ANAND et al 2011).

Su e colaboradores (2014) demonstraram que, no modelo experimental de traumatismo craniano induzido por perfusão lateral em ratos, o tratamento com taurina na dose de 200 mg/kg, imediatamente após a lesão e diariamente ao longo de 7 dias por injeção intravenosa, foi capaz de melhorar a recuperação funcional, diminuindo os níveis de expressão de GFAP (proteína fibrilar ácida glial), e várias citocinas como IFN-α (interferon-α), IL-1β, IL-4, IL-5, IL-6, IL-10 e TNF-α. Legitimando o achado principal de vários estudos acima citados, a taurina na dose de 100 mg/kg também foi capaz de prevenir a toxicidade espermática e testicular induzida pelo decanoato de nandrolona (esteróide anabólico) em ratos. Sendo este efeito protetor, relacionado às suas atividades antioxidantes, anti-inflamatórias e anti-apoptóticas, demonstradas através da diminuição da expressão dos níveis de TNF-α, produção de NO, e reduziu o dano ao DNA, bem como o aumento da atividade de SOD, e níveis de glutationa reduzida (AHMED, 2015).

Estudos *in vitro* e *in vivo* demonstram que a taurina está apresenta efeitos benéficos sobre o sistema reprodutor (YANG et al., 2017; ADEDARA et al., 2018). *In vitro*, o papel protetor da taurina foi demonstrado sobre espermatócitos de ratos em cultura celular, expostos a radiação ionizante (raios-γ) e comparados quanto a administração prévia de taurina. Em doses de 20, 40, 80 mM, a taurina foi responsável por atenuar a citotoxicidade, além de diminuir a produção de ROS e a taxa apoptótica das células (YANG et al., 2017). Além disso, estudos anteriores revelaram ainda que a atividade antioxidante da taurina melhorou a disfunção reprodutiva em ratos com

hipertensão induzida. Os grupos tratados com taurina obtiveram normalização das enzimas antioxidantes superóxido dismutase, catalase e glutationa peroxidase encontradas nos testículos e no epidídimo, além de apresentarem aumento no número de espermatozoides e na motilidade dos mesmos (ADEDARA et al., 2018).

Em relação a reprodução em fêmeas, Mu e colaboradores (2015) investigaram o papel deste aminoácido sobre a secreção de seis hormônios, durante os estágios do ciclo estral. O estudo analisou ratas fêmeas durante 60 dias, sendo elas divididos em 5 grupos: grupo controle (C), grupo taurina 1% em água (T1), grupo taurina 2% em água (T2), grupo β-alanina 1% em água (β1), grupo β-alanina 2% em água (β2). O nível do hormônio folículo-estimulante (FSH) sofreu um aumento não significativo, já o hormônio liberador de gonadotrofina (GnRH) teve aumento significativo no grupo T2 (em metaestro). O hormônio luteinizante (LH) encontrou-se aumentado apenas em proestro no grupo T2, a prolactina (PRL) encontrou-se aumentada em todas as fases do ciclo, nos grupos T1 e T2 quando comparados ao grupo controle. O estradiol (E2) teve seus níveis aumentados nos animais em proestro e estro em ambos os grupos (T1 e T2). Concluindo, portanto, que os efeitos da taurina na reprodução feminina pode ser alcançado principalmente regulando a atividades de hormônios relacionados ao eixo hipotálamo-hipófise-ovário.

1.4 Álcool

A substância lícita mais comumente utilizada e, a terceira causa de morte no mundo é o álcool (KOOB e MOAL, 2005). Dentre os seus principais consumidores encontram-se os adolescentes na faixa etária entre 15-25 anos cujo, consumo médio é de 112 g por dia (DAWSON et al, 2008; KIRBY e BARRY, 2012). Algumas evidências na literatura indicam uma alta relação entre o consumo na adolescência e posterior abuso de álcool na vida adulta (WILCOX et al, 2014). Além disso, no período da adolescência o cérebro está em processo de maturação que requer mudanças em sua neurotransmissão e em sua plasticidade sináptica, o que é acompanhado de mudanças estruturais em regiões específicas (hipocampo, córtex pré-frontal e sistema límbico) (DAHL, 2004). Todas estas evidências sugerem que o cérebro adolescente pode ser mais vulnerável aos efeitos maléficos do álcool (SLAWECKI et al, 2004), como a intoxicação alcoólica que resulta em dano e morte cerebral, em processos de estresse oxidativo e inflamação (WHITE et al, 2000; OBERNIER et al, 2002). Por tais motivos, uma avaliação de álcool associado

ou não a bebidas energéticas sobre diversos parâmetros comportamentais, oxidativos e imunológicos se faz tão necessária nesta população.

As revisões na literatura que abordam sobre o consumo de álcool em adolescentes retratam alterações na substância cinzenta do cérebro tanto em áreas corticais quanto subcorticais, sendo que estas alterações diminuem o volume do córtex pré-frontal, hipocampo e amígdala. Tais alterações cerebrais causam danos em funções neurocognitivas como a memória e o aprendizado (JACOBUS e TAPERT, 2013). Avaliando os efeitos do álcool no organismo, ratos Wistar machos e fêmeas, em população adolescente (28-56 dias) (0,5; 1 e 2 g/kg) e adulta (65-102 dias), foram tratados com álcool (0,5; 1 e 3,5 g/kg) em garrafas de livre escolha através do teste *Conditioned Taste Aversion* (CTA). Os resultados encontrados foram que ratos adultos tem maior aversão ao álcool e consomem menores quantidades, porém ao longo do tempo os adolescentes diminuem o consumo enquanto os adultos aumentam, e que quanto maior a dose de álcool maior será a aversão condicionada ao gosto, tanto em adolescentes quanto adultos (SAPYTA et al, 2014). Em trabalhos da literatura que avaliam o efeito da abstinência de álcool, Zahr e colaboradores (2016), submeteram ratos Wistar a 4 dias de tratamento com álcool (5 g/kg) seguido de 10 dias de abstinência, e os resultados encontrados foram opostos aos relatados anteriormente, visto que não houve incremento do consumo de álcool após a abstinência, ou dano cerebral persistente, quantificado por método de imagem *in vivo*, assim como não houve dano hepático.

O álcool ingerido em grupos mais vulneráveis pode causar alteraçãoes imunológicas significativas, Pascual et al (2014) observou que 3 g/kg de álcool via intraperitoneal, por um período de 2 semanas, em ratos adolescentes (30 dias) causa o aumento da expressão de de receptores de reconhecimento de padrões como o Receptor *Tool Like* (TLR4 e TLR2), envolvidos na ativação de células da imunidade inata e consequente produção de citocinas inflamatórias (TNF-α e IL-1β), assim como desintegração da bainha de mielina dos neurônios do córtex pré-frontal, contribuindo para o déficit cognitivo comportamental. Assim como o experimento anterior, Alfonso-Loeches e colaboradores (2013) também avaliaram os efeitos de receptores *Tool-Like*, após administração de álcool (10% misturado a água na garrafa) em camundongos machos e fêmeas C57BL/6 WT ($TLR4^{+/+}$) e C57BL/6 KO ($TLR4^{-/-}$ *knockout*) por um período de 5 meses. Ao final da exposição observou-se que o álcool causou a maior expressão de enzimas inflamatórias (iNOS e COX-2), citocinas (IL-1β, TNF-α), ativação da caspase-3 e perda neuronal do córtex cerebral dos animais de ambos os sexos, porém

os camundongos do sexo feminino tendem a ter os maiores níveis nos parâmetros avaliados, sendo que os animais nocautes de TLR4 foram protegidos contra os efeitos deletérios do álcool em ambos os gêneros.

No período da adolescência, importantes transformações podem ser influenciadas pelo consumo de bebidas alcoólicas. O aumento do hormônio liberador de hormônio luteinizante (LHRH), que promove a secreção das gonadotrofinas hipofisárias, responsáveis por estimular a secreção de esteroides gonadais para maturação dos órgãos reprodutivos, também sofre ação do álcool, através da alteração na expressão de genes que regulam esse aumento (DEES et al., 2017). Em meninas, o atraso no desenvolvimento puberal, principalmente das mamas e da menarca, é quatro vezes maior naquelas que fazem uso de álcool (PECK et al., 2011).

O consumo de álcool por adolescentes aumenta o risco de alteração de função neuroendócrina, potencialmente modificando o tempo de desenvolvimento pubertal. Os estudos demonstram que o álcool atua no hipotálamo alterando a expressão e função de genes excitatórios e inibitórios e neuro-hormônios relacionados à puberdade, que são críticos para o aumento oportuno da secreção de LHRH (hormônio liberador do hormônio luteinizante) e o início da puberdade (DEES et al, 2017).

Os efeitos negativos sobre o sistema reprodutivo masculino, associados ao uso do álcool, também são relatados. Diminuição na massa dos testículos e órgãos sexuais secundários, baixos níveis séricos de testosterona e impotência erétil se relacionam à exposição crônica de ratos machos à bebidas alcoólicas (EMANUELE e EMANUELE, 2001; MUTHUSAMI e CHINNASWAMY, 2005). Outros estudos avaliando o potencial tóxico na reprodução feminina sugerem que a ingestão de álcool de modo excessivo causa toxicidade reprodutiva marcante, tal como diminuição da fertilidade, obstrução da ovulação, e redução da massa dos ovários. Devido à ocorrência de eventos apoptóticos nas células granulosa causando redução da viabilidade celular (LIU et al., 2018).

1.5 Protocolo *binge drinking*

O protocolo do tipo *binge drinking* é identificado pelo consumo excessivo de álcool em curto período de tempo, atingindo um nível de concentração sanguíneo de 0,08 g/dL, correspondendo a 5 ou mais doses em homens e 4 ou mais doses em mulheres, em um período de 2 horas (National Institute on Alcohol Abuse and Alcoholism-NIAAA). Caracterizando-se entre intoxicação alcoólica intensa e períodos de abstinência em dias

alternados (MAURAGE et al, 2020). Este tipo de protocolo é muito comum em adolescentes e adultos jovens em países do Ocidente (LEES et al, 2018). Um estudo conduzido na Europa revelou que adolescentes entre os 18 e 19 anos são a principal faixa etária de consumo alcoólico do tipo BD (*binge drinking*), principalmente nos países do oriente europeu (DORMAL et al, 2019). A faixa etária de consumo deste tipo de protocolo varia entre os estudos, porém muitos entram em consenso que começa ao redor dos 13 anos de idade e que sua prevalência aumenta na adolescência e chega ao pico na idade adulta (18-22 anos) (ADAN et al, 2016).

Segundo a revisão de Carbia e colaboradores (2018) o consumo do tipo BD é associado ao déficit de memória verbal e em funções executivas, principalmente a um controle inibitório deficiente, porém a atenção, velocidade, memória de curto prazo e construção visuoespacial parecem não ser afetadas. De acordo com Siqueira e colaboradores (2015), o álcool é a substância de maior abuso por crianças e adolescentes nos Estados Unidos sendo causa de morte e ferimentos graves nesta idade (acidente de carro, homicídio e suicídio). Sendo que os consumidores do tipo BD tendem a ser pessoas extrovertidas, impulsivas e em busca de grandes emoções, mas também podem se tornar mais estressadas, ansiosas e deprimidas, devido ao consumo excessivo de álcool (KUNTSCHE et al, 2017). Em relação a diferença de consumo entre os sexos, de acordo com dados da literatura, os homens tendem a consumir o álcool (tipo BD) em maior nível do que as mulheres e estas diferenças entre os sexos aumenta com a idade (12-20 anos) (CHUNG et al, 2018).

1.6 Bebidas energéticas, cafeína, taurina e álcool avaliados sobre diversos parâmetros

Um protocolo na literatura que mimetiza o consumo feito por humanos chamado de *Binge Drinking* (3 dias de tratamento por semana) é utilizado como modelo animal de estudo, o qual pode-se dar de maneira aguda (1 semana) ou de forma subcrônica (4 semanas) (LAUING et al, 2008). Neste sentido, Fagundes et al (2016) avaliaram o efeito deste protocolo, sobre as glândulas salivares parótidas e submandibulares, em ratas fêmeas Wistar (35 dias) em dois grupos de estudo, um exposto a 1 semana de tratamento com álcool (3 g/kg) e outro grupo exposto ao mesmo tratamento, porém ao longo de 4 semanas. Os autores demonstraram que não houve diferença entre os grupos em relação

a concentração de nitritos na parótida e submandibular, mas houve aumento significativo dos níveis de malondialdeído (MDA) nas respectivas glândulas.

Costa Valle (2018) avaliou a neurotoxicidade subcrônica oral de bebida energética em ratos, bem como seus respectivos constituintes: cafeína e taurina, isoladas e em associação. Os resultados demonstraram que os grupos tratados com cafeína (3,2 mg/kg) e taurina (40 mg/kg) (doses equivalentes ao consumo de 3 latas), tiveram melhor desempenho nos testes de memória de curta duração. Entretanto, observou-se um desbalanço na atividade das enzimas antioxidantes: superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx) no córtex pré-frontal, hipocampo e estriado, principalmente nos grupos tratados com a associação de cafeína e taurina. Além do aumento na produção de espécies reativas de oxigênio no córtex pré-frontal e hipocampo, principalmente nos ratos tratados com a associação de cafeína e taurina. Porém, os grupos tratados com energético não apresentaram diferença nos níveis de enzimas antioxidantes. Neste estudo, em todos os testes foi evidente que a associação de cafeína e taurina, em concentrações semelhantes a maior dose de energético, diferiu dos efeitos da administração apenas do energético.

Na literatura, poucos estudos foram encontrados avaliando impacto de energéticos no sistema antioxidante. ZEIDÁN-CHULIÁ e colaboradores (2013) avaliaram cafeína, taurina e guaraná sobre o sistema antioxidante em células humanas neuronais SH-SY5Y e demonstraram que houve redução dos níveis basais de geração de radicais livres. A combinação de cafeína ou taurina com guaraná induziu uma diminuição na atividade da superóxido dismutase (SOD) *in vitro*, assim como a associação de cafeína ou taurina ao guaraná diminui a atividade da catalase (CAT) nas células, porém não houve mudanças na atividade da glutationa peroxidase (GPx).

Os efeitos de taurina, cafeína, e energéticos associados ao álcool também tem sido investigado sobre diferentes aspectos. Neste contexto, o consumo diário por 4 semanas de energéticos da marca Red Bull® (1,5 mL/100g) por ratos Wistar causou significativa hepatotoxicidade com alterações histopatológicas e em enzimas hepáticas (ALT e AST), além de perda da arquitetura e necrose de hepatócitos (KHAYYAT et al., 2012). Em adição, verificou-se que o tratamento com energéticos (3,75 mL/kg e 7,5 mL/kg) associadas ao álcool (1,0 g/kg e 2,0 g/kg) em ratos Wistar por 30 dias elevaram os níveis de uréia, creatinina, enzimas hepáticas, bilirrubina total e potássio (UGWUJA, 2014). Referente ao efeito desta associação em relação a marcadores de inflamação e parâmetros

oxidativos, Díaz e colaboradores (2016) demonstraram que a exposição de ratos Wistar machos (de 90 dias) a associação de álcool (2,5 g/kg) e energético (7,5 mL/kg) por 60 dias aumentou a gliose reativa e expressão proteica de IL-1β, TNF-α, iNOS, geração de espécies reativas de oxigênio e peroxidação lipídica. Reis e colaboradores (2017) avaliaram o consumo de energéticos (3,5 g/kg e 7 g/kg) associados ou não ao consumo de álcool (1 g/kg) sendo o tratamento por via oral ao longo de 14 dias, através da formação de espécies reativas de oxigênio (ROS) nas medidas de SOD, CAT, GSH, MDA (malondialdeído) e análises histopatológicoas nos órgãos fígado e cérebro. A conclusão do estudo mostrou que tanto os energéticos quanto o álcool causou dano oxidativo e peroxidação lipídica nas estruturas analisadas, porém o efeito danoso foi pior quando ocorreu sua associação.

Os testes de comportamento animal também são avaliados em trabalhos na literatura, associando álcool e bebidas energéticas. Krahe e colaboradores (2017) utilizaram camundongos Swiss (40 dias) em grupo tratados com álcool (4 g/kg 40%) e energético (8 mL/kg), separados e em associação, em testes de locomoção e coordenação motora (*open-field* e *rota-rod*, respectivamente). Os resultados encontrados foram que a associação de álcool e bebida energética aumenta a atividade locomotora, porém aumenta a ansiedade e demonstra piores desempenhos de coordenação motora.

Em humanos, a administração conjunta de álcool e energético, em população adolescente, interfere nos sintomas estimulantes e de depressão. Sendo que, quanto maior a ingesta de energéticos (acima de 3 latas) maiores serão os efeitos estimulantes e os efeitos da intoxicação alcoólica (DROSTE et al, 2017). O efeito do consumo de álcool (3,4 g/kg) associado ou não ao energético (10,71 mL/kg) por 6 dias consecutivos também foi avaliado em testes comportamentais (teste de atividade locomotora, teste de reconhecimento de objeto, teste de discernimento social e teste de condicionamento de preferência por lugar). Os achados encontrados foram que a associação de energético ao álcool não previne subsequentes danos cognitivos, e em maior grau sua associação aumenta o comportamento de busca pelo álcool, ou seja, a associação de bebida energética ao álcool pode estar relacionada ao abuso e dependência alcoólica (TAKAHASHI et al, 2015).

Camundongos tratados via i.p com cafeína (1, 5, 15, 30 mg/kg), taurina (30, 60, 300, 600 mg/kg) e etanol (1,75, 2,5, 3,25 g/kg) tiveram seu desempenho avaliado em teste de locomoção (Open Field). Os testes foram realizados de forma aguda e 19 dias após administrações (com apenas cafeína), e ao final com as combinações de etanol e taurina.

Os resultados encontrados revelarem que a cafeína aumenta a atividade locomotora e produz um efeito aditivo na atividade locomotora induzido pelo etanol. Além disso, embora não tenha um efeito substancial na locomoção por si só, ou em combinação com o etanol, a taurina pode potencializar ainda mais esse efeito em doses específicas (ULENIUS et al, 2019).

Os achados disponíveis na literatura relacionam o efeito do álcool, cafeína, taurina, e energéticos de forma isolada no organismo, porém a sinergia de todos estes componentes não é explorada. O álcool possui inúmeros achados quanto a sua toxicidade. A cafeína e a taurina a possível proteção a danos oxidativos e imunológicos. No entanto o resultado de sua administração ao longo do tempo, e em protocolo descontínuo ainda não foram descobertos. Este estudo teve como justificativa averiguar a associação de álcool e energéticos, assim como os constituintes da bebida energética (cafeína e taurina), sobre parâmetros hematológicos, bioquímicos, imunológicos, histológicos, comportamentais e reprodutivos em ratos Wistar machos e fêmeas durante a puberdade.

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3 OBJETIVOS

3.1 Objetivo geral

Considerando o aumento progressivo do consumo de energéticos associados ao álcool, o pouco conhecimento acerca do impacto toxicológico deste consumo excessivo e a escassez de dados científicos sobre os efeitos da associação cafeína-taurina-álcool, este estudo teve como objetivo avaliar a toxicidade de bebida energética, seus constituintes cafeína e taurina, isolados e em associação ao álcool em ratos Wistar pré-púberes em protocolo descontínuo de administração repetida do tipo *binge drinking*

3.2 Objetivos específicos

Avaliou a toxicidade de bebida energética, dos constituintes cafeína e taurina associados ou não ao álcool, em protocolo *binge drinking* de administração em ratos Wistar pré-púberes (28-60 dias) machos e fêmeas;

Avaliou o efeito da bebida energética, cafeína, taurina e álcool em protocolo *binge drinking* de administração sobre os parâmetros bioquímicos de ureia, creatinina, alanina transaminase (ALT), aspartato transaminase (AST) em ratos Wistar pré-púberes (28-60 dias) machos (artigo 1);

Avaliou o efeito da bebida energética, cafeína, taurina e álcool em protocolo *binge drinking* de administração sobre os parâmetros hematológicos através de hemograma completo e contagem diferencial de série branca de ratos Wistar pré-púberes (28-60 dias) machos (artigo 1);

Avaliou o efeito da bebida energética, cafeína, taurina e álcool em protocolo *binge drinking* de administração sobre a produção de citocinas pró-inflamatórias TNF-α, IL-1β, ROS e óxido nítrico no plasma de ratos Wistar pré-púberes (28-60 dias) machos (artigo 1);

Avaliou o efeito da bebida energética, cafeína, taurina e álcool em protocolo *binge drinking* de administração sobre avaliação histopatológica em fígado, rim e adrenal de ratos Wistar pré-púberes (28-60 dias) machos (artigo 1);

Avaliou o efeito da bebida energética, cafeína, taurina e álcool em protocolo *binge drinking* de administração sobre o comportamento de ratos Wistar pré-púberes (28-60 dias) machos e fêmeas, por meio dos testes *open field*, *pluz maze* e reconhecimento de objetos (artigo 2);

Avaliou o efeito da bebida energética, cafeína, taurina e álcool em protocolo *binge drinking* de administração sobre a regularidade de ciclo estral, massa relativa dos órgãos sexuais, e qualidade dos oócitos quanto a sua morfologia e maturação em amostras de ratas Wistar fêmeas pré-púberes (28-60 dias) (artigo 2);

Avaliou o efeito da bebida energética, cafeína, taurina e álcool em protocolo *binge drinking* de administração sobre parâmetros reprodutivos de massa relativa dos órgãos sexuais, número de espermátides e espermatozoides, e avaliação morfológica dos espermatozoides, em amostras de ratos Wistar machos pré-púberes (28-60 dias) (artigo 2);

4 ARTIGO 1

Artigo a ser submetido para à Revista Toxicology and Applied Pharmacology (*IF*: 4.21).

Components of energy drinks, caffeine and taurine, reduce immunological damage even when associated with alcohol: a Binge Drinking protocol in male prepubertal Wistar rats

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Abstract

The consumption of energy drinks reaches an increasing number of adolescents and young adults. Its consumption associated with alcohol has been high among young people. The aim of this study was to evaluate the effects of alcohol, energy drink, and its constituents (caffeine and taurine), associated or isolated in a Binge Drinking protocol on proinflammatory cytokines production, histological evaluation (kidney and liver), hematological, biochemical and oxidative parameters. Male Wistar rats (prepubertal) were treated from the 32nd day of life by gavage using the Binge Drinking protocol (3 consecutive days, with an interval of 4 days per week, for 4 weeks) with energy drink (10 mL/kg), caffeine (3.2 mg/kg), taurine (40 mg/kg) or alcohol (2 g/kg, 20%), combined or isolated. The results revealed an increase in the production of inflammatory cytokines (TNF-α and IL-1β) most in the groups treated with energy drinks, alcohol, and alcohol associated with energy drinks. As well as the increment presence of histopathological changes in liver and kidneys (vacuolization, cellular infiltrate, and hydropic degeneration), mainly in groups treated with alcohol. The results suggest harmful effects of alcohol and energy drink, in a discontinuous protocol of repeated exposure to prepubertal Wistar rats, which should be investigated in the medium and long term on the various biological systems.

Keywords: energy drinks associations, adolescents, alcohol abuse, experimental study, cytokines.

1. Introduction

The consumption of energy drinks has grown exponentially in the last decades and reached an extraordinary number of consumers around the world (Bailey et al., 2014). The adolescents are being the main consumers and the target of marketing strategies responsible for moving this economic market (Howland et al., 2013). The formulations containing several components are intended to increase the state of alertness, attention, and energy boost (Pennay et al., 2012). The main component is caffeine, which is present in large quantities, and the others are taurine, vitamins, and sugars (Ishak et al., 2012). In some formulations it is possible to observe the presence of *ginseng*, *Ginko biloba*, guarana and glucuronolactone (Sanctis et al., 2017; Higgins et al., 2010).

The multi billionaire industry of energy drinks grow every day and as result numerous health agencies create guidelines for the protection of children and adolescents. Mainly because they can be in contact with large quantities of these excitatory components (Zucconi et al., 2013). Countries such as Denmark, Turkey, Norway, Uruguay, Switzerland, Lithuania, Latvia and Iceland have banned or restricted the sale to minors (under 18 years of age) (NIHID, 2013; Australian Food News, Reyes et al., 2015).

Energy drinks cause health problems related to neurological and cardiac effects (Curran and Marczinski, 2017), which generates major medical emergencies mainly in more vulnerable conditions (cardiac patients, with sleep disorders, anxious and stressed) (Ali et al., 2015). In addition to the intake of energy drinks, it is common to mix alcohol by adolescents and young adults, which leads to an increase in alcohol consumption and more severe intoxications (Caviness et al., 2017). This is due to the effects of alcohol intoxication being masked by the stimulating effect of the caffeine present in energy drinks, but the deleterious effects continue to exist (Roemer and Stockwell, 2017).

The binge drinking is very common in adolescents and young adults and is characterized by greater consumption of alcohol on some days of the week. According to Caetano and collaborators in partnership with the INPAC institute (2013), in Brazil, about 40% of men and 18% of women report consumption in this way. However, alcohol in critical periods of development can alter the plasticity and maturation of neural cells, which can cause behavioral and cognitive damage (Guerri and Pascual, 2010).

Several issues related to the toxic effects of the associated consumption of alcohol and energy drinks remain unclear, especially among adolescents, as well as the type of effect in relation to different forms of consumption. This study aimed to evaluate the toxic effect of discontinuous and repeated exposure of energy drinks and their main constituents (caffeine and taurine) associated or not with alcohol, through the Binge Drinking protocol during puberty in Wistar rats on proinflammatory cytokines production, histological evaluation (kidney and liver), hematological, biochemical and oxidative parameters.

2. Materials and methods

2.1 Animals

The project was approved by the Ethics Committee on the Use of Animals (CEUA) (221/17) of the Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA). All experiments followed NIH guidelines for the use and care of experimental animals. Male Wistar rats (N = 100), with 28 days were bred and kept in the bioterium of UFCSPA, in groups of 3 to 4 animals per box, under ideal temperature conditions (22 ± 2º C) with a 12-hour dark light cycle (from 7 a.m. to 7 p.m.), and with free access to water and food.

2.2 Binge Drinking protocol

The Binge Drinking protocol consisted of the oral administration (gavage) of different solutions containing alcohol for 3 consecutive days, once a day, for 4 weeks, with intervals of 4 days a week from the 32nd day of life (at 28 days the animals started getting used to the researcher). The period of life chosen to administer the animals was adolescence (28 days old in rats) to mimic the consumption in humans. The animals were divided into 10 groups of 5 animals per group and were treated orally for 28 days with energy drink 10 mL/kg, caffeine 3.2 mg/kg, taurine 40 mg/kg, caffeine 3.2 mg/kg + taurine 40 mg/kg, distilled water 10 mL/kg, alcohol 20% (2 g/kg) (control ethanol), alcohol 20% (2 g/kg) + energy drink (10 mL/kg), alcohol 20% (2 g/kg) + caffeine 3.2 mg/kg, alcohol 20% (2 g/kg) + taurine 40 mg/kg and alcohol 20% (2 g/kg) + caffeine 3.2 mg/kg + taurine 40 mg/kg. The dose of energy drink administered to the animals was equivalent to the consumption of 3 cans of 250 mL of energy drink by an adult man with an average weight of 75 kg, thus, each animal in the energy drink group received 10 mL/kg of the respective commercial product ©Red Bull. The doses of the caffeine (3.2

mg kg) (Sigma Aldrich, Brazil), taurine (40 mg/kg) (Sigma Aldrich, Brazil) and association groups were equivalent to those present in 10 mL of the energy drink (Ferreira et al., 2013). The alcoholic solution (ethanol P.A) administered had a concentration of 20%, equivalent to doses of 2 g/kg. Caffeine, taurine and alcohol were solubilized in distilled water. In all experiments, distilled water was used as a control and administered by gavage, as well as the other treatments, obeying the maximum volume of 10 mL/kg.

2.3 Sample preparation

The animals were euthanized on the 60th day and evaluation proinflammatory cytokines production, histological evaluation (kidney and liver), biochemical and oxidative parameters. The animals were euthanized by guillotine, without previous anesthesia, since the use of anesthetic could interfere in the analysis of the activity of antioxidant enzymes and in the production of inflammatory cytokines, in the central nervous system (Delogu et al., 2004; Yesilkaya et al., 1998; Turkan et al., 2004). The structures were dissected and frozen at -80º C until further analysis. The Flowchart Experimental design (Figure 1) shows the treatment groups under study, as well as the Binge Drinking protocol, and the analyzes that were performed.

2.4 Hematological and biochemical evaluations

Hematological analyses were performed using blood collection using the hematology analyzer (Micros-60). The erythrogram counted: platelets, red blood cells, hematocrit (HCT), hemoglobin (HGB), mean corpuscular volume (CMV), mean corpuscular hemoglobin (HCM) and mean hemoglobin concentration (CHCM). The leukogram evaluated the total leukocyte count and the number of monocytes, lymphocytes, basophils, neutrophils and eosinophils.

Biochemical analyses were assessed using markers of renal function (serum urea and creatinine), liver function (alanine aminotransferase (ALT), aspartate

aminotransferase (AST) and alkaline phosphatase (FA)) and cardiac muscle damage marker (creatine phosphokinase mb fraction (CKMB)). The serum quantifications were performed using commercial kits (DGKC Bioclin®), according to the manufacturer's instructions, in an X BS-120 spectrophotometer.

Hematological and biochemical assessments were developed as complementary tests and are presented in tables on supplementary data.

2.5 Histological analyses

The organs (liver, kidneys and adrenals) were fixed in 10% buffered formaldehyde for histological analysis. After standard histological preparation of the tissues, the paraffin blocks were sectioned into 3 µm fragments, which were mounted on a slide, stained with hematoxylin/eosin and observed under an optical microscope at 40, 100 and 400x. Histopathological analyses was conducted by total screening of each slide in search of changes in tissue structure (cell degenerations, congestion, infiltrate), which are characterized by severity scores (mild (focal), moderate (1/3) and severe (2/3), according to the proportion in relation to the tissue sample. At the end of the evaluation of each slide (per organ), the scores for each change were presented as median and interquartile intervals, as well as the sum of changes per organ, for each experimental group (N= 5/group).

2.6 Systemic cytokine levels

The systemic levels of interleukin-1β (IL-1 β) and tumor necrosis factor α (TNF-α) were evaluated by Enzyme Linked Immunosorbent Assay (ELISA) following the manufacturer's recommendations (Peprotech Inc., EUA). The intra-assay coefficient was < 7.5%. The detection limits of each cytokine were: IL-1 β, 9 – 1000 pg/mL; TNF- α 4 – 500 pg/mL.

2.7 Reactive oxygen and nitrogen species

The systemic levels of reactive oxygen species (ROS) were evaluated through the fluorescence intensity of the redox-sensitive dye 2′,7′-dichlorodihydrofluorescein diacetate (DCFH, 100 μM, Sigma-Aldrich) (excitation and emission wavelengths of 480 and 535 nm, respectively) using SpectraMax M2e (Molecular Devices, USA). The systemic nitrite levels were measured as a metabolite of nitric oxide in the plasma by the Griess method as previously described by Miranda and coworkers (2001).

2.8 Statistical analysis

The normality of the data was assessed by the Shapiro-Wilk test, with the data with normal distribution being presented as mean + standard deviation and the statistical analysis performed by one-way ANOVA or repeated measures. Qualitative data and/or that did not have a normal distribution are presented in medians and interquartile intervals and evaluated by the Kruskal Wallis test. All analyses were performed using SPSS Statistics version 23.

3. Results

3.1 Hematological and biochemical analyses

As general parameters to evaluate the alterations induced by discontinuous protocol of repeated exposure to alcohol and energy drinks, associated or isolated in young rats. It was observed the effects of the exposure on hematological parameters as an indicator of systemic damage and biochemical parameters as indicator of muscle cardiac damage, liver, and kidneys enzymes. There was no statistical difference in the hematological parameters evaluated (WBC, RBC, HCT, HGB, VCM, HCM, CHCM and PLT) ($p > 0.05$, one-way ANOVA or Kruskal Wallis) (Appendix 1), nor in the biochemical parameters (ALT, AST, FA and CKMB) ($p > 0.05$, one-way ANOVA or Kruskal Wallis) (Appendix 2)

3.2 Histological analysis

To analyze the possible effects of discontinuous protocol of repeated exposure of alcohol and energy drinks, associated or isolated in young rats. The renal and hepatic tissue was selected to evaluate the possible toxic effects of alcohol on the body, isolated and associated with energy drinks. Since these tissues are responsible for filtering and eliminating toxins. To observe possible stress effects (cortisol liberation) caused by alcohol, the adrenal tissue was analyzed. In general, it was observed significant alterations including congestion, hemorrhage, hydropic degeneration, hyaline degeneration, vacuolization, and cellular infiltration. Of these, the most common are vacuolization, cellular infiltration, and hydropic degeneration. In these analyses, the isolated scores of each alteration were compared and also the sum of the scores of all alterations present in each evaluated organ.

The liver tissue showed a statistical difference in relation to the presence of cellular infiltrate (alcohol and alcohol + caffeine + taurine groups were different from the

control group) ($p < 0.001$, Kruskal-Wallis) (Table 1). Hydropic degeneration and vacuolization were the most common changes in the sum of the scores (in the groups: alcohol, alcohol + energy drink, alcohol + caffeine and alcohol + caffeine + taurine compared to the control) ($p < 0.0001$, Kruskal-Wallis). The letter B on the figure 2 showed the liver on the group treated with alcohol + caffeine + taurine, and similar results were found in the treated groups (Figure 2).

The renal evaluation showed a significant difference in relation to the sum of the vacuolization and cellular infiltrate ($p < 0.0001$, Kruskal-Wallis), as we can see in the letter D on kidney in the group treated with alcohol. The groups: alcohol, alcohol + energy drink, alcohol + caffeine, alcohol + taurine and alcohol + caffeine + taurine was different from the control (Figure 2). However, the scores separately did not show difference in vacuolization and cellular infiltrate (Table 1).

In relation to adrenal tissues, although we did not find any significant difference between the groups in relation to the isolated alterations on vacuolization and cellular infiltrate, there was a difference in sum of changes ($p < 0.0001$, Kruskal-Wallis) for groups of caffeine, alcohol + taurine and alcohol + caffeine + taurine in relation to control animals (Appendix 3). In the figure on the appendix 3 it's possible to see the group treated with alcohol + caffeine + taurine, that had similar histological as the other groups.

3.3 Cytokine, nitric oxide and reactive oxygen species determination

The systemic levels of IL-1β were increased in the animals treated with energy drink, alcohol, alcohol + energy drink and alcohol + caffeine + taurine compared to the animals of control group ($p < 0.0001$, ANOVA post-hoc Bonferroni) (Figure 3). TNF-α also was increased in the serum of animals administrated with energy drink, alcohol, and alcohol + energy drink ($p < 0.0001$, Kruskal-Wallis) (Figure 3). The association of alcohol

229 with caffeine or taurine reduced the production of pro-inflammatory cytokines in relation
230 to animals treated with alcohol and/or energy drinks.

231 The production of nitric oxide was different in the alcohol and alcohol + energy
232 drink groups compared to the control ($p < 0.005$, ANOVA post-hoc Bonferroni) (Figure
233 3). However, the production of reactive oxygen species was not different between groups
234 ($p = 0.67$, Kruskal-Wallis) (Figure 3).

235 4. Discussion

236 The discontinuous protocol of repeated exposure of energy drinks and in
237 association with alcohol was able to induce histopathological changes in the liver, kidneys
238 and adrenals, mainly generating vacuolization and cellular infiltration in groups treated
239 with alcohol. The systemic levels of IL-1β were increased in the animals from groups of
240 energy drinks, alcohol, alcohol associated with energy drinks and alcohol associated with
241 caffeine and taurine compared to the control group. In addition, TNF-α was increased in
242 serum of energy drink, alcohol and alcohol associated with energy drinks. Among these
243 findings, it is important to emphasize that the association of alcohol with caffeine and
244 taurine separately was able to decrease the production of pro-inflammatory cytokines,
245 close to the values found in the control group. However, the administration of the
246 components did not influence the hematological and biochemical parameters. In general,
247 the results of hematological and biochemical parameters found in our study were like the
248 values described of controls of others bioteriuns described in other studies (Lima et al.,
249 2014; Melo et al., 2012).

250 The literature finds discrepancies regarding the influence of energy drinks and
251 alcohol on blood parameters, in relation to our findings. The Red Bull® energy drink
252 administrated (1.5 mL/100g) daily for 4 weeks in Wistar rats, caused significant
253 hepatotoxicity with histopathological changes and liver enzymes (ALT and AST) and the

loss of architecture and necrosis of hepatocytes in rats (Khayyat et al 2012). Ugwuja et al. (2014) found that the treatment with energy drinks (3.75 mL/kg and 7.5 mL/kg) associated with alcohol (1.0 g/kg and 2.0 g/kg) in Wistar rats, for 30 days, increased the levels of urea, creatinine, liver enzymes, total bilirubin, and potassium. In our study it was not find hematological or biochemical alterations, the difference among the studies may be due to the type of protocol the age of animals and the doses administered.

The Binge Drinking protocol administration was able to generate alterations in the liver and kidneys. The main changes observed were vacuolization, cellular infiltrate, and hydropic degeneration. In agreement with our previous study, the main groups affected were those treated with alcohol and its associations. Even without evaluating the acute effect of the substances, the discontinuous repetition protocol, caused equal changes in animals treated with the same doses and components. In the previous study, where adult male Wistar rats were treated acutely (orally) with alcohol (20% 2g/kg), energy drink (10 mL/kg), or their main components: caffeine (3.2 mg/kg) and taurine (40 mg/kg), associated or isolated. The results showed that alcohol and the association with energy drinks caused liver damage (congestion, hemorrhage, hydropic degeneration, and hyaline degeneration) and kidney damage (hyaline degeneration and hemorrhage) (Costa Valle et al., 2018).

Moreover, the literature confirms that alcohol causes toxicity, and that the main organ affected is the liver. Male and female Sprague-Dawley rats submitted to Binge Drinking protocol (3 doses in the interval of 12 h) with alcohol (5 g/kg body weight) presented hepatic steatosis (fat vesicles) (Shukla et al., 2019).

The kidney is also an organ greatly affected by excessive alcohol consumption, causing problems in the maintenance of body fluids, electrolytes, acid-base control and even blood pressure (Epstein, 1997). “Semi-voluntary” alcoholism (only available liquid)

(15% ethyl alcohol) to Wistar rats (50 days) for 12 weeks was able to induce the formation of vacuoles in the renal tubules and reduce the space of the Bowman's capsule (Oliveira et al., 2011). In another study with 90-day-old animals (Wistar rats) treated for 24 weeks with alcohol (50 mL at 20%), daily in the drinking bottle, the histological analyses revealed the presence of hydropic degeneration, hyaline degeneration, and macro fat vesicles in the liver of the animals. An increase in the glomeruli was observed in the kidney, with a lack of characterization of the Bowman's capsule space, proliferation of mesangial cells, presence of hyperemia and regions of intense inflammation with the presence of T lymphocytes (Sousa et al., 2018). Our results corroborate the literature data showing liver and kidney histopathological damage induced even in a discontinuous administration protocol. Carried out after a period greater than 24 hours (5 days after the last administration), it was possible to observe significant tissue damage in liver and kidney. A fact to be highlighted is the age of the animals, which are adolescents and are in development, which causes greater concern.

The isolated energy drink also generates tissue damage, in this sense, according to Mansy and collaborators (2017) Red Bull © (1.1/100 g and 2.2 mL/100 g daily) for 12 weeks induced damages to the hepatic tissue (congestion, inflammation and fibrosis) and renal tissue (congestion, inflammation, degeneration and necrosis). Moreover, energy drink (3.57 mL/kg) administered via gavage for 8 weeks to adult male Sprague-Dawley rats caused congestion, hemorrhage, and necrosis in the renal tissue (Bano et al., 2020). The dose administered in these studies is lower than that used in our study, but the administration period is longer (our experiments were for 4 weeks).

Regarding to the pro-inflammatory cytokines, in this study an increase in the production of TNF-α was found in groups treated with energy drinks, alcohol and alcohol associated with energy drinks. Moreover, the production of IL-1b was also increased in

these same groups and in the group treated with alcohol associated with caffeine and taurine. Díaz et al. (2016) demonstrated that the exposure of male Wistar rats (90 days) to the association of alcohol (2.5 g/kg) and energy drink (7.5 mL/kg) for 60 days increased the proinflammatory cytokines of IL-1β, TNF-α. Pascual et al (2014) observed that 3 g/kg of alcohol intraperitoneally administrated to prepubertal rats (30 days) for a period of 2 weeks caused significant augment in the expression of Toll Like Receptor (TLR4 and TLR2) in the prefrontal cortex, with subsequent increase in the production of TNF-α and IL-1β in the brain. Another study demonstrated the isolated energy drinks also induced the augment in IL-1β and IL-6 levels in serum of adult rats treated with Code Red © brand at concentrations of 0.72 mL/100 g/day and 1.44 mL/100 g/day for 8 weeks (Alansari, 2020).

It is important to point out that the association of alcohol and caffeine or alcohol plus taurine was able to inhibit the production of pro-inflammatory cytokines induced by alcohol). These findings can be related to the fact that caffeine and taurine can act as anti-inflammatory agents. Confirming these results, the administration of taurine (1g/kg) per day for 6 weeks in adult male Wistar rats, was capable to decrease the expression of IL-1β and TNF-α in liver, however in the groups treated with alcohol (3g/kg) the production was increased (Jen-Lin, et al. 2015). The administration of (2% w/v) associated with alcohol (6 g/kg) for 60 days was able to decrease the production of pro-inflammatory cytokines (IL-1β and TNF-α) in adult male Wistar rats compared to alcohol alone (Devi et al., 2010). Caffeine has also been studied as a protector against immune damage due to alcohol intake. In a study conducted with Kunming mice, alcohol intake (5% v / v), orally over 8 weeks, associated with caffeine (at doses 5, 10 and 20 mg / kg) was able to decrease the proinflammatory cytokines (IL-1β and TNF-α) in liver tissue, demonstrating that caffeine has a protective role against alcoholic liver damage (Lv et al., 2010).

5. Conclusion

Our data indicate that the discontinuous and repeated exposure of the energy drinks associated with alcohol causes damage in young animals, which are in critical period of development (puberty). Alcohol associated with energy drinks increased the production of cytokines (TNF-α and IL-1β), indicating a possible participation of inflammatory cytokines in the tissue damage. In contrast, caffeine and/or taurine associated with alcohol decrease these parameters. These data show that the excessive consumption of alcohol and energy drinks need caution, especially in adolescents and young adults who ingest these components in the manner addressed by the Binge Drinking protocol.

Conflict of interest

The authors declare that are no conflict of interest to disclose.

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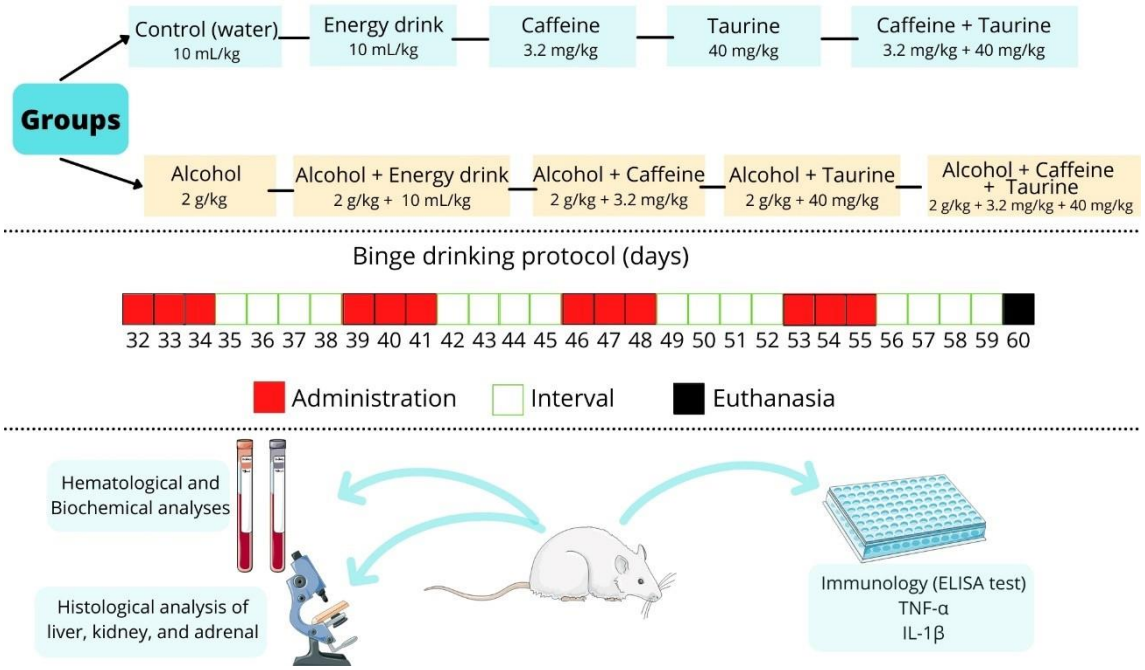

Figure 1: Flowchart of experimental design.

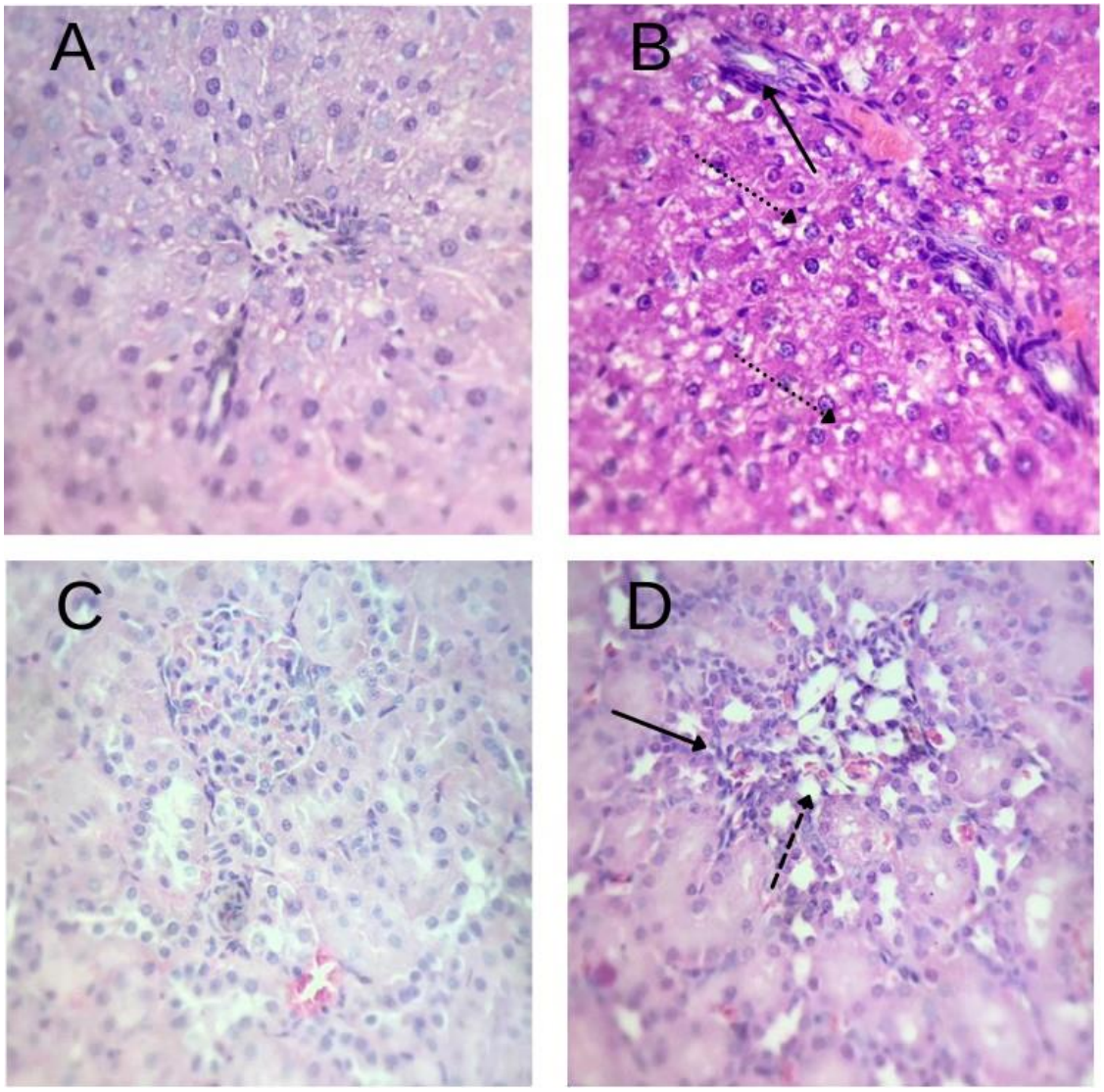

Figure 2: Effects of alcohol, energy drink and its constituents (caffeine and taurine) isolated or associated on the liver and kidneys. The data of the isolated changes (AI) and the sum of the changes (SA) were evaluated by Kruskal — Wallis. Liver (AI, $p < 0.001$); SA, $p < 0.001$), kidneys (AI, $p > 0.05$; SA, $p < 0.001$). The black lines show cellular infiltrate, the dotted lines indicate hydropic degeneration, and the dashed lines indicate vacuolization. The letters A and B represent the liver, C and D kidney. The letters A, C are control slides, letter B (alcohol + caffeine + taurine) treated group, and letter D (alcohol) treated groups. 400x magnification.

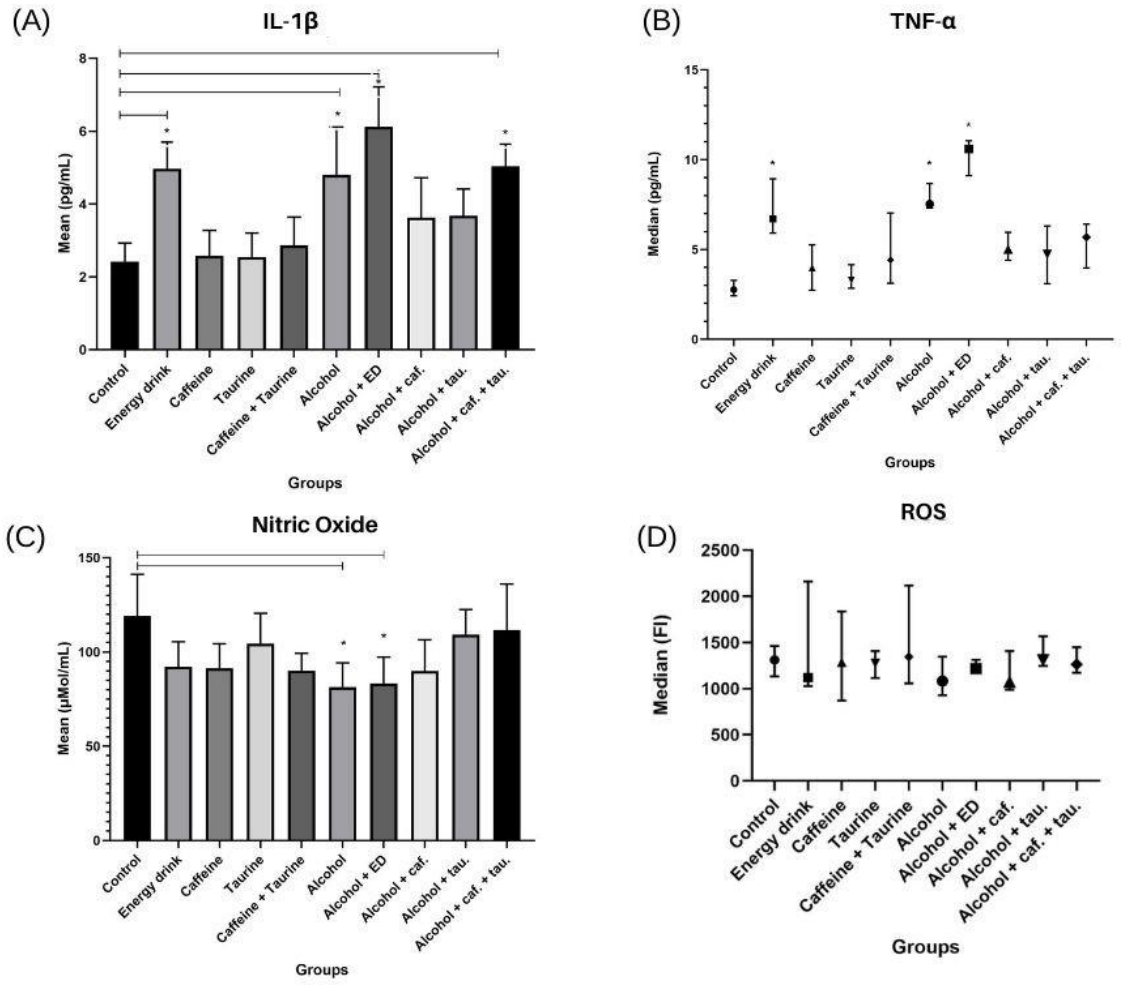

Figure 3: Effects of alcohol, energy drink and its constituents (caffeine and taurine) isolated or associated on IL 1β (A) ($p < 0.0001$, ANOVA post-hoc Bonferroni), TNF-α (B) ($p < 0.0001$, Kruskal-Wallis), nitric oxide (C) ($p < 0.005$, ANOVA post-hoc Bonferroni), reactive oxygen species (ROS) ($p = 0.67$, Kruskal-Wallis). The asterisk (*) represent the difference between the groups in relation to the control group. The abbreviations mean: ED-energy drink; Caf-caffeine; Tau-taurine. N= 5 animals/group

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Table 1: Histopathological changes in the liver and kidney of male prepubertal Wistar rats treated with energy drink and alcohol in Binge Drinking protocol

	Liver			Kidney			
Groups	Hydropic degeneration	Vacuolization	Cellular infiltrate	Sum	Vacuolization	Cellular infiltrate	Sum
Control	2 (1;2)	0 (0;0)	2 (1;2)	2 (1;2)	1 (1;1)	(1;1)	(3;4)
Energy drinks	2 (2;3)	0 (0;1)	3 (1;3)	3 (2;3)	1 (0;1)	(1;2)	(4;5)
Caffeine	2 (1;3)	0 (0;0)	2 (1;2)	2 (1;2)	1 (0;1)	(1;1)	(2;4)
Taurine	3 (2;3)	0 (0;1)	1 (1;1)	1 (1;2)	1 (1;1)	(1;1)	(4;5)
Caffeine + taurine	3 (1;3)	0 (0;0)	2 (1;2)	2 (1;3)	1 (0;1)	(1;2)	(4;5)
Alcohol	2 (2;2)	1 (0;1)	3 (2;3)	3 (3;4) *	1 (1;2)	(2;3) *	(5;6) *
Alcohol + energy drinks	2 (2;3)	0 (0;1)	3 (2;3)	3 (3;4) *	1 (1;1)	(1;3)	(5;6) *
Alcohol +caffeine	2 (2;2)	1 (0;1)	3 (3;3)	4 (3;4) *	1 (1;2)	(2;2)	(5;7) *
Alcohol + taurina	2 (1;2)	1 (0;1)	3 (3;3)	3 (3;4) *	1 (1;1)	(2;3)	(5;5) *
Alcohol + caffeine + taurine	3 (2;3)	1 (1;1)	3 (2;3)	3 (3;4) *	1 (1;1)	(2;3) *	(6;7) *

Nonparametric data are represented by medians and interquartile ranges. The sum represents the summation of the scores in the histopathological analysis p sent in prese tissue. The asterisks (*) represent the statistical differences in the sum of the changes in the liver and kidneys ($p < 0.0001$, Kruskal-Wallis),
as well as in well as in the infiltrate of the hepatic tissue ($p < 001$, Kruskal-Wallis). N= 5 animals/group.

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Supplementary Materials

Appendix 1: Hematological parameters of male prepubertal Wistar rats treated with energy drink and alcohol in Binge Drinking protocol

Groups	WBC	RBC	HGB	HCT	MCV	MCH	MCHC	PLT
Control	8.9 ± 1.0	7.50 (7.34; 7.74)	14.80 (14.70; 15.20)	41.88 ± 1.33	55.00 (55.00; 56.00)	19.86 ± 0.62	35.8 ± 1.37	704.00 (702.00; 745.00)
Energy drinks	7.3 ± 0.9	7.22 (7.13; 7.46)	14.70 (14.20; 14.80)	39.04 ± 3.99	55.00 (54.00; 56.00)	19.74 ± 0.70	35.74 ± 1.57	769.00 (747.00; 819.00)
Caffeine	7.4 ± 0 .9	7.09 (7.06; 7.34)	14.70 (14.30; 15.00)	40.6 ± 1.80	56.00 (56.00; 56.00)	20.02 ± 0.64	35.86 ± 1.40	822.00 (803.00; 832.00)
Taurine	6.3 ± 1.2	7.48 (7.11; 7.73)	14.60 (14.40; 15.00)	41 ±2.35	55.00 (54.00; 55.00)	19.48 ± 1.17	35.52 ± 1.69	777.00 (631.00; 797.00)
Caffeine + taurine	6.4 ± 1.8	7.38 (7.32; 7.58)	14.65 (14.35; 14.75)	41.7 ±1.05	56.00 (54.50; 57.00)	19.55 ± 0.78	34.92 ± 1.41	759.00 (712.50; 812.50)
Alcohol	8.4 ± 1.3	7.25 (7.18; 8.06)	14.90 (14.50; 15.40)	42.5 ± 3.82	55.00 (55.00; 57.00)	19.66 ± 1.08	35.2 ± 2.09	769.00 (738.00; 849.00)
Alcohol + energy drinks	9.2 ± 2.6	7.54 (7.23; 8.11)	15.00 (14.20; 15.70)	42.8 ± 3.85	56.00 (55.00; 56.00)	19.58 ± 0.44	35.28 ± 0.99	784.00 (742.00; 784.00)
Alcohol + caffeine	7.7 ± 0.9	7.88 (7.82; 8.00)	15.30 (14.80; 15.50)	43.36 ± 2.15	55.00 (54.00; 55.00)	19.16 ± 0.70	35.1 ± 1.28	747.00 (711.00; 802.00)
Alcohol + taurine	8.1 ± 1.1	7.84 (7.37; 8.06)	14.60 (14.10; 15.00)	42.64 ± 2.88	55.00 (54.00; 56.00)	18.94 ± 0.68	34.5 ± 1.45	751.00 (698.00; 846.00)
Alcohol + caffeine + taurine	8.5 ± 1.9	7.76 (7.38; 8.04)	14.90 (14.90; 15.70)	43.56 ± 3.09	55.00 (55.00; 56.00)	19.32 ± 1.03	34.9 ± 1.55	707.00 (701.00; 734.00)

Parametric data are represented by mean ± standard deviation (p> 0.05, one-way ANOVA) and nonparametric data are represented by medians (interquartile intervals) (p> 0.05, Kruskal-Wallis). The abbreviations are represented by: leukocytes (WBC), erythrocytes (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), concentration of mean corpuscular hemoglobin (MCHC) and platelets (PLT). N = 5 animals/group

S5u4p8plementary Material

Appendix 2. Biochemical parameters of male prepubertal Wistar rats treated with energy drink and alcohol in Binge Drinking protocol

Groups	Urea	Creatinine	AST	ALT	Phosphatase	Ckmb
Control	52.00 (48.00; 55.00)	37.00 (36.00 38.00)	167.00 (82.00; 207.00)	70.00 (25.00; 76.00)	202.00 (188.00; 228.00)	13737.8 ± 7498.26
Energy drinks	43.00 (42.00; 54.00)	32.00 (20.00; 35.00)	167.00 (144.00; 169.00)	86.00 (61.00; 87.00)	238.00 (189.00; 251.00)	10139.4 ± 5537.19
Caffeine	49.00 (44.00; 54.00)	35.00 (34.00; 39.00)	162.00 (54.00; 174.00)	55.00 (19.00; 77.00)	244.00 (191.00; 264.00)	13104.4 ± 2953.43
Taurine	46.00 (45.00; 46.00)	35.00 (32.00; 36.00)	203.00 (176.00; 233.00)	72.00 (72.00; 80.00)	192.00 (145.00; 244.00)	13249.0 ± 7998.8
Caffeine+ taurine	44.00 (40.50; 47.00)	32.00 (29.50; 36.00)	63.00 (6.50; 173.50)	54.00 (23.00; 95.00)	29.50 (12.00; 220.50)	7587.75 ± 9084.6
Alcohol	46.00 (43.00; 49.00)	37.00 (31.00; 38.00)	213.00 (150.00; 216.00)	57.00 (34.00; 64.00)	195.00 (178.00; 230.00)	16797.8 ± 11769.5
Alcohol + energy drinks	50.00 (49.00; 51.00)	36.00 (32.00; 40.00)	83.00 (46.00; 94.00))	47.00 (6.00; 56.00)	176.00 (152.00; 208.00)	12545.4 ± 4352.1
Alcohol + caffeine	45.00 (40.00; 46.00)	35.00 (35.00; 36.00)	121.00 (41.00; 193.00)	69.00 (68.00; 80.00)	188.00 (172.00; 227.00)	12668.4 ± 8924.3
Alcohol + taurine	46.00 (43.00; 50.00)	37.00 (35.00; 40.00)	157.00 (104.00; 179.00)	64.00 (63.00; 69.00)	177.00 (163.00; 226.00)	11672.6 ± 3225.5
Alcohol + caffeine + taurine	45.00 (45.00; 47.00)	34.00 (32.00; 38.00)	179.00 (178.00; 206.00)	69.00 (65.00; 77.00)	193.00 (165.00; 196.00)	14558.8 ± 4228.7

Parametric data are represented by mean ± standard deviation (p> 0.05, one-way ANOVA) and nonparametric data are represented by medians (interquartile intervals) (p> 0.05, Kruskal-Wallis). The abbreviations are represented by: transaminase alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatinine phosphokinase MB fraction (CKMB). N = 5 animals / groups

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552 **Supplementary Materials**
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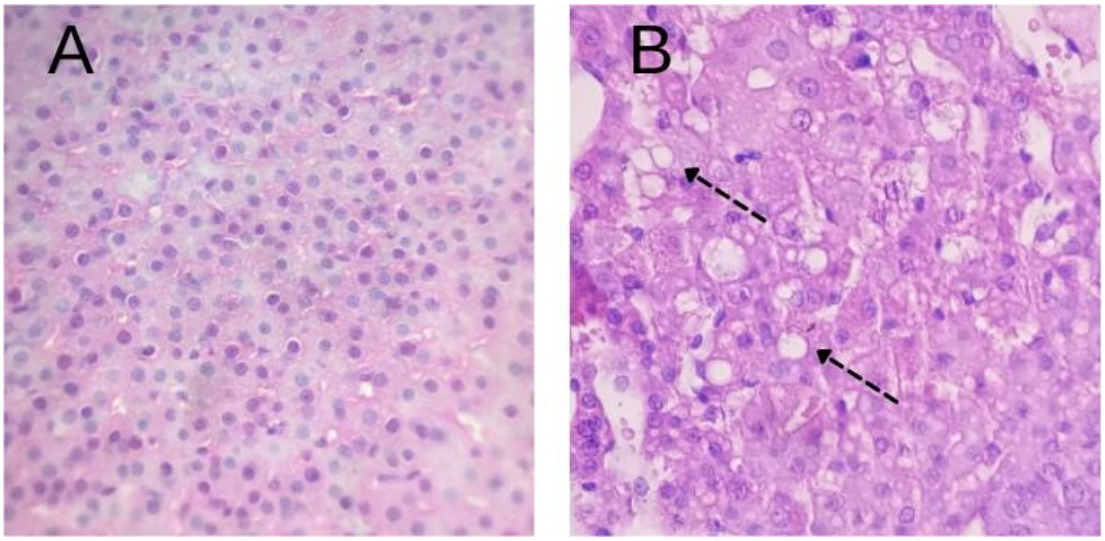

554 **Appendix 3**: Effects of alcohol, energy drink and its constituents (caffeine and taurine) isolated or associated on adrenals. The data
555 of the isolated changes (AI) and the sum of the changes (SA) were evaluated by Kruskal-Wallis, adrenal (AI, $p > 0.05$; SA, p
556 <0.001).The dashed lines indicate vacuolization. The letters A and B represent the adrenals. The letter A are control slide and the
557 letter B treated group (alcohol + caffeine + taurine). 400x magnification.
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5 ARTIGO 2

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Energy drinks and alcohol in a binge drinking protocol in Wistar rats: Male and female behavioral and reproductive effects

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Energy drinks and alcohol in a binge drinking protocol in Wistar rats: Male and female behavioral and reproductive effects

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ABSTRACT

The consumption of energy drinks is common among adolescents and young adults. The possible effects (mainly behavioral and reproductive) of ingestion in this population remain unknown. For this reason, this study aimed to evaluate the behavioral and reproductive effects of energy drinks and their main constituents (caffeine and taurine), as well as their combinations with alcohol, via a binge drinking protocol in male and female Wistar rats during puberty. In this study, 100 male and 100 female rats were treated with a binge drinking protocol 3 days a week over 4 weeks from postnatal day (PND) 28 to PND 60, which included 10 mL/kg by oral gavage of distilled water, energy drink, caffeine (3.2 mg/kg), taurine (40 mg/kg), and their combinations with alcohol (2 g/kg). The animals were evaluated by behavioral tests from PND 56 to PND 60 (open field, plus maze and object recognition) and reproductive parameters (estrous cycle regularity, weight of sexual organs, oocyte quality, spermatid and sperm count, sperm morphology and testosterone level). Locomotor activity was increased in females in the groups combined with alcohol (except alcohol + caffeine) and in the caffeine group. Long-term memory was increased in males in the caffeine and taurine groups even when combined with alcohol. The combination of energy drinks and alcohol did not have significant effects on the reproductive parameters of either sex of rats during puberty. We concluded that energy drinks (and their main constituents) and alcohol combinations did not cause alterations in reproductive profiles, and locomotor activity and long-term memory were increased in females and males, respectively.

1. Introduction

Energy drinks act as energizers or stimulants, improving concentration and providing well-being, which usually attracts a young population (Ballistreri and Corradi-Webster, 2008). However, a study revealed that the majority (64.3 %) of students who use energy drinks are not aware of the possible side effects (insomnia, anxiety, tachycardia, and gastrointestinal disturbances) (Scuri et al., 2018). In addition, since 2000, the consumption of energy drinks has been combined with alcohol (Pennay and Lubman, 2012). This combination can cause damage, since the stimulating effect of caffeine antagonizes the sedative effects of alcohol. Consequently, there has been an increase in alcohol consumption, leading to greater toxicity (Hahn et al., 2012; Howland et al., 2011; Ferreira et al., 2004) and a higher risk of accidents and violence (Berger et al., 2013; Quigley et al., 2019), including heightened susceptibility to alcohol dependence (Mendonça et al., 2018).

Despite the positive effects of energy drink consumption (memory, humor, and vigilance) (Alsunni, 2015), some adverse effects are important to mention. According to a review by Nadeem et al. (2021) of the pediatric population (age range, 11–19 years) in 10 studies (a total of 89,836 individuals), the most frequent adverse effects were palpitations (17.5 %), abdominal pain (14.5 %), muscle soreness (14.4 %),

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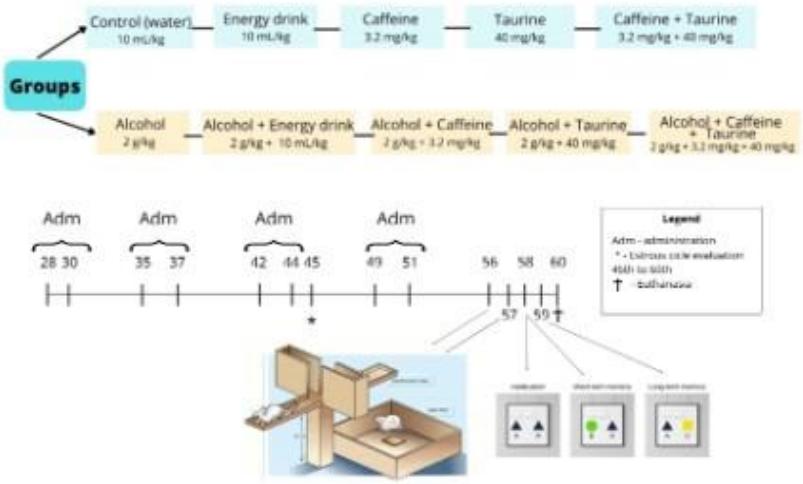

Fig. 1. Timeline of administration and behavioral testing.

headaches (20.9 %), insomnia (35.4 %), increased urination (16.4 %), and stress (35.4 %).

The possible neurological effects of alcohol in adolescents are harmful, indicating a decrease in the volume of structures in the prefrontal cortex, hippocampus, and amygdala. Such brain changes impair neurocognitive functions such as memory and learning (Jacobus and Tapert, 2013). Furthermore, during adolescence, the brain is undergoing a maturation process that requires changes in its neurotransmission and synaptic plasticity, which is accompanied by structural changes in specific regions (hippocampus, prefrontal cortex, and limbic system) (Dahl, 2004). The combined administration of alcohol and energy drinks in an adolescent population interferes with stimulating effects (heart palpitations, insomnia, fidgeting, tremors, agitation, energy peak, fast speech, and muscle tension) and depressive effects (nausea, dizziness, headache, walking difficulties, slurred speech, fatigue, and decreased coordination) (Droste et al., 2017).

Regarding the reproductive system, the hormonal dysregulation caused by alcohol interferes with menstrual cycle regularity, decreases fertility, and causes hypogonadism (Rachdaoui and Sarkar, 2017). Some studies show that caffeine works in men and women, reducing fertility in both sexes. In cases in which consumption exceeds 699 mg/day, the fertility rate can be reduced by 44 % (Sadeu et al., 2010). However, in vitro and in vivo studies have demonstrated that taurine has beneficial effects on the reproductive system (Yang et al., 2017; Adedara et al., 2018). From the data collected in the literature, this study aims to evaluate the effects of caffeine, taurine, and alcohol intake on the male and female reproductive systems.

Considering the progressive increase in the consumption of energy drinks combined with alcohol and the lack of knowledge about the toxicological impact of this excessive consumption, the objective of this study was to evaluate the effects of energy drinks and their main constituents (caffeine and taurine), both in isolation and in combination with alcohol, on animal behavior and reproductive parameters in prepubertal rats (males and females).

2. Materials and methods

2.1. Animals

This project was approved by the Ethics Committee on the Use of Animals (CEUA) of the Federal University of Health Sciences of Porto Alegre (UFCSPA) under protocol number 221/17. All experiments followed the National Institutes of Health (NIH) guidelines for the use and care of experimental animals. Male and female Wistar rats at postnatal day (PND) 28 were housed in the bioterium of UFCSPA in groups of 3/4 animals per cage under ideal temperature conditions (22 ± 2 °C) with a dark/light cycle of 12 h (7 am to 7 pm) and with free access to water and food for behavioral (N = 200) and reproductive analyses (N = 100).

2.2. Binge drinking protocol

The binge drinking protocol consisted of a large volume administered in a short period of time. This study comprised a protocol with an oral administration (gavage) of solution (energy drinks, caffeine, taurine, alcohol, water, and their combinations) once a day for 3 consecutive days, with intervals of 4 days, for 4 weeks starting on PND 28 (Fig. 1). The animals were divided into 10 groups (10 animals per group in the behavioral analysis and 5 animals per group in the reproductive analysis) and were treated for 28 days with 10 mL/kg by oral gavage of distilled water, energy drink, caffeine, taurine, or their combinations (Fig. 1). The dose of energy drink was equivalent to the consumption of 3 cans of 250 mL by an adult with an average weight of 75 kg. Thus, each animal in the energy drink group received 10 mL/kg of the commercial product Red Bull©. The doses of caffeine (3.2 mg kg) (Sigma Aldrich, Brazil), taurine (40 mg/kg) (Sigma Aldrich, Brazil) and combination group solutions were equivalent to those contained in 10 mL of the energy drink (Ferreira et al., 2013). The alcoholic solution (ethanol p.a.) administered had a concentration of 20 % ethanol, which is equivalent to a dose of 2 g/kg. Caffeine, taurine, and alcohol were solubilized in distilled water.

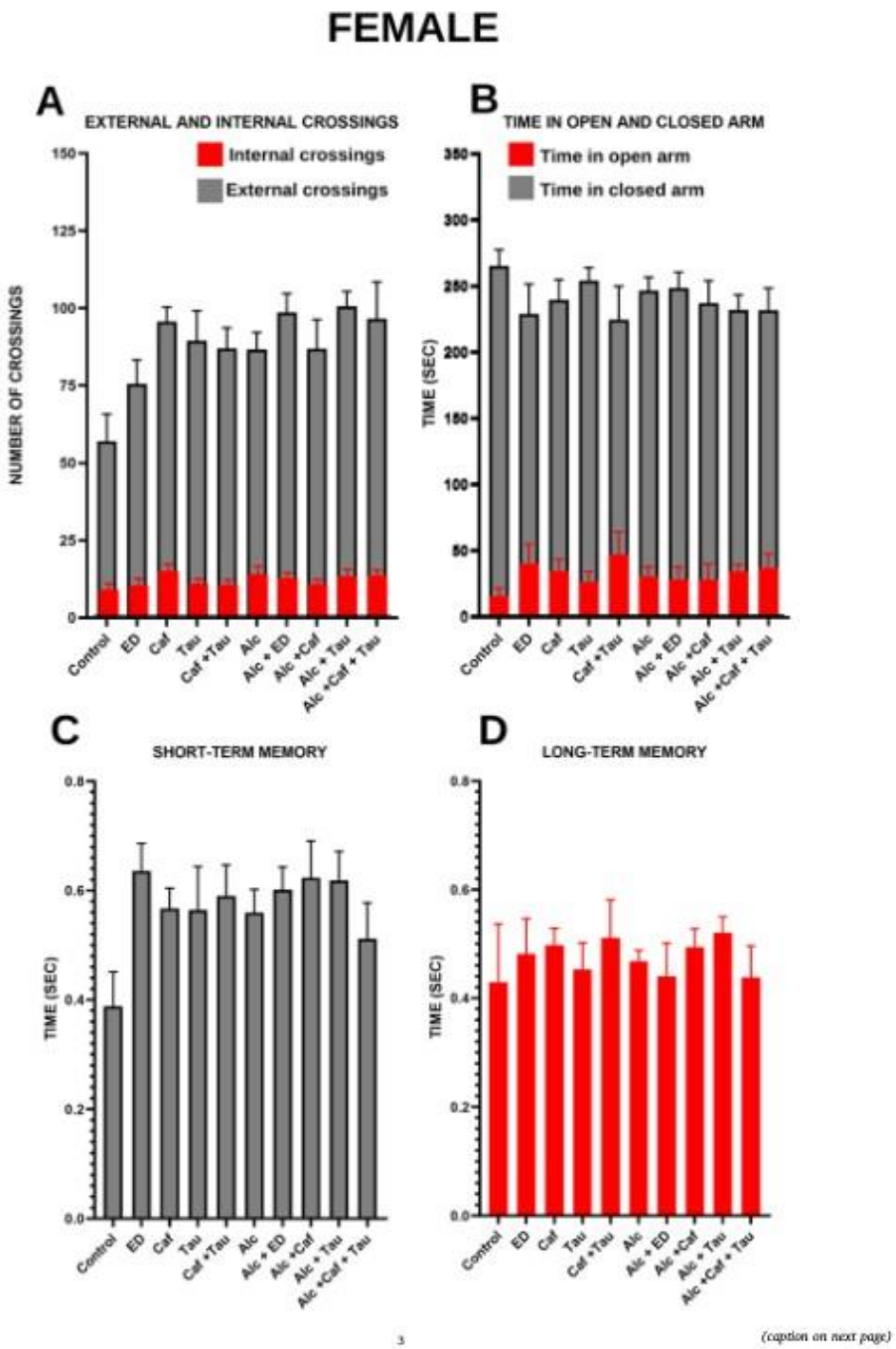

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Fig. 2. Effects of alcohol, energy drinks and their constituents (caffeine and taurine), isolated or combined, in the behavioral analysis of the binge drinking protocol in female Wistar rats (N = 100). A shows the open field test, B shows the plus maze test, and C and D show the object recognition test. Parametric data were assessed by one-way ANOVA (post hoc Bonferroni) ($p = 0.02$), and the results are expressed as the mean ± SEM. The asterisk (*) represents the difference between the groups in relation to the control group. Abbreviations: ED—energy drink; Caf—caffeine; Tau—taurine and Alc—alcohol.

2.3. Behavioral analysis

2.3.1. Open field test

The open field arena was intended to quantify general spontaneous locomotor activity on PND 56. Behavioral assessment was performed in a black acrylic arena measuring 60 × 60 × 30 cm, divided into 16 squares. The rats were gently placed in the posterior left corner and were monitored to observe their exploratory profile for five minutes. The number of external crossings (when the animal moved into any of the squares in the peripheral area of the apparatus) was associated with locomotor activity; latency to drop out of the first quadrant was a measure to assess anxiety, as were the internal crossings (when the animal moved within the central area of the apparatus). The number of rearings (number of times the animal stood up on its hind legs) and grooming time (time spent cleaning itself) were also evaluated (Pinoger et al., 1992; Silveira et al., 2005) (Fig. 1).

2.3.2. Elevated plus maze test

The apparatus consisted of a cross-shaped wooden labyrinth, with each of the four arms (2 closed and 2 open) measuring 50 cm long by 10 cm wide, and contained a central square measuring 12 × 12 cm. The animals were placed in the center of the cross maze with the head facing the closed arm. Their behavior was recorded during a 5-min exposure period on PND 56, and the parameters evaluated were the number of entries into the open and closed arms, the length of stay in each arm and head dipping (Almeida et al., 2006; Knapp and Breese, 2012) (Fig. 1). The apparatus was intended to analyze the anxiety profile.

2.3.3. Object recognition test

The object recognition test was performed in the same arena as the open field test. The procedure consisted of 3 phases: habituation, exposure, and discrimination. In the habituation phase, the animals could explore the environment for 5 min for two consecutive days. In the exposure phase, 24 h after habituation, 2 identical objects (A1 and A2) were placed on opposite sides in the corners of the arena, which the rats could explore for 5 min. After a period of 90 min, an identical familiar object (A3) and a new object (B) were placed in the same place where A1 and A2 had previously been, allowing the rat to explore these new objects for another 5 min (short-term memory) on PND 58. After a period of 24 h, the familiar object (A3) and a new object (C) were placed in the arena, and the animal could explore these new objects for another 5 min (long-term memory) on PND 59. Object exploration was defined as the rat approaching the object within approximately 2 cm or less, as well as when the animal touched the object with its vibrissae. The analyses were performed using the following parameters: the total time exploring the new object (TN), divided by the total time exploring the two objects; the new (TN) and the familiar (TF) in the discriminatory phase TN / (TF + TN) (Maciel et al., 2014) (Fig. 1).

2.4. Sample collection

The animals were euthanized on PND 60 in the same period, and reproductive organs were removed to perform the analyses. The animals were euthanized by guillotine, without prior anesthesia, as the use of anesthetic could interfere with the analysis of the activity of antioxidant enzymes and the production of inflammatory cytokines, which were used as complementary data from the experimental 'n' (Delagu et al., 2004; Yesilkaya et al., 1998; Türkan et al., 2004).

2.5. Reproductive evaluation

2.5.1. Female rats

Reproductive analyses in females were performed for estrous cycle regularity, the relative mass of the ovaries, and the quantity and quality of oocytes. The rats were evaluated daily for regularity of their estrous cycle (including the number of estrus events and the number of days in the estrogenic phase — proestrus plus estrus) from PND 45–60. Immediately after euthanasia, rat ovaries were collected, weighed, transferred to a plate containing phosphate-buffered saline (PBS) (37 °C), and supplemented with 2 % fetal bovine serum (FBS), in which scarification of the ovarian cortex was performed to release the CCOs (*Cumulus-oocyte complexes*). The CCOs were selected according to the appearance of the cumulus cells and the homogeneity of the ooplasm under a stereomicroscope. The CCOs were classified into 4 groups (with grades 1 and 2 considered appropriate; grade 3, intermediate; and grade 4, degenerated): Grade 1 — CCOs with homogeneous ooplasm and several layers of cumulus cells with a high degree of compaction; Grade 2 — CCOs with homogeneous ooplasm and fewer cumulus cell layers and less compaction; Grade 3 — CCOs with more heterogeneous ooplasm, presence of granulation, with few or no layers of uncompacted cumulus cells; Grade 4 — CCOs with unusual shapes and very heterogeneous ooplasm, not showing cumulus cell layers.

2.5.2. Male rats

Reproductive analyses were performed to assess the relative mass of the following organs: testicle, right and left epididymis, seminal vesicle, and prostate. Additionally, the number of spermatids and sperm, morphological assessment of sperm, and testosterone level were evaluated. One testicle of each male was then released from the tunica albuginea, dipped in 10 mL of 0.9 % sodium chloride and 0.05 % Triton X-100 solution, and manually homogenized to count spermatids. One hundred microliters of the homogenate was diluted in 900 μL of 0.9 % sodium chloride solution. After dilution, the number of spermatids resistant to homogenization was counted in a Neubauer chamber. In the chamber, ¼ of the area of each reticulum was counted (total 0.1 mm^3). The resulting value of the spermatid count of each testicle was multiplied by the dilution factor used (10^2) and by the correction factor of the chamber (10^2) and adjusted to cm^3 ($\times 10^2$). Thus, the total value counted results in millions of spermatids ($\times 10^6$). In addition, the tail of the epididymis was processed, similar to the testis, for the sperm count. The tail was isolated from the rest of the organ, sectioned into small parts, dipped, and homogenized in the same volume and dilution used for counting spermatids. Sperm were also counted in a Neubauer chamber. The same multiplication factor (10^6) was applied. Furthermore, to perform the morphological evaluation of sperm, the right and left vas deferens of each male were washed with 1 mL of 0.9 % sodium chloride solution, and the smear was mounted on a glass slide with a drop of the wash mixed with a drop of 2 % eosin. The pathological spermatic evaluation was realized under an optical microscope at 400× (Olympus-C011), and the pathologies included head defects (malformed, small, detached) and/or tail defects (short, curled, folded) (Dallegrave, 2003).

2.6. Liquid chromatography-tandem mass spectrometry analysis for testosterone analysis

Samples were prepared in a 1.5 mL tube by the addition of 100 μL of plasma and 400 μL of acetonitrile, followed by agitation and then centrifugation at 9000 ×*g* for 6 min. The supernatant (400 μL) was transferred to a new tube, and the extraction was performed with 1 mL

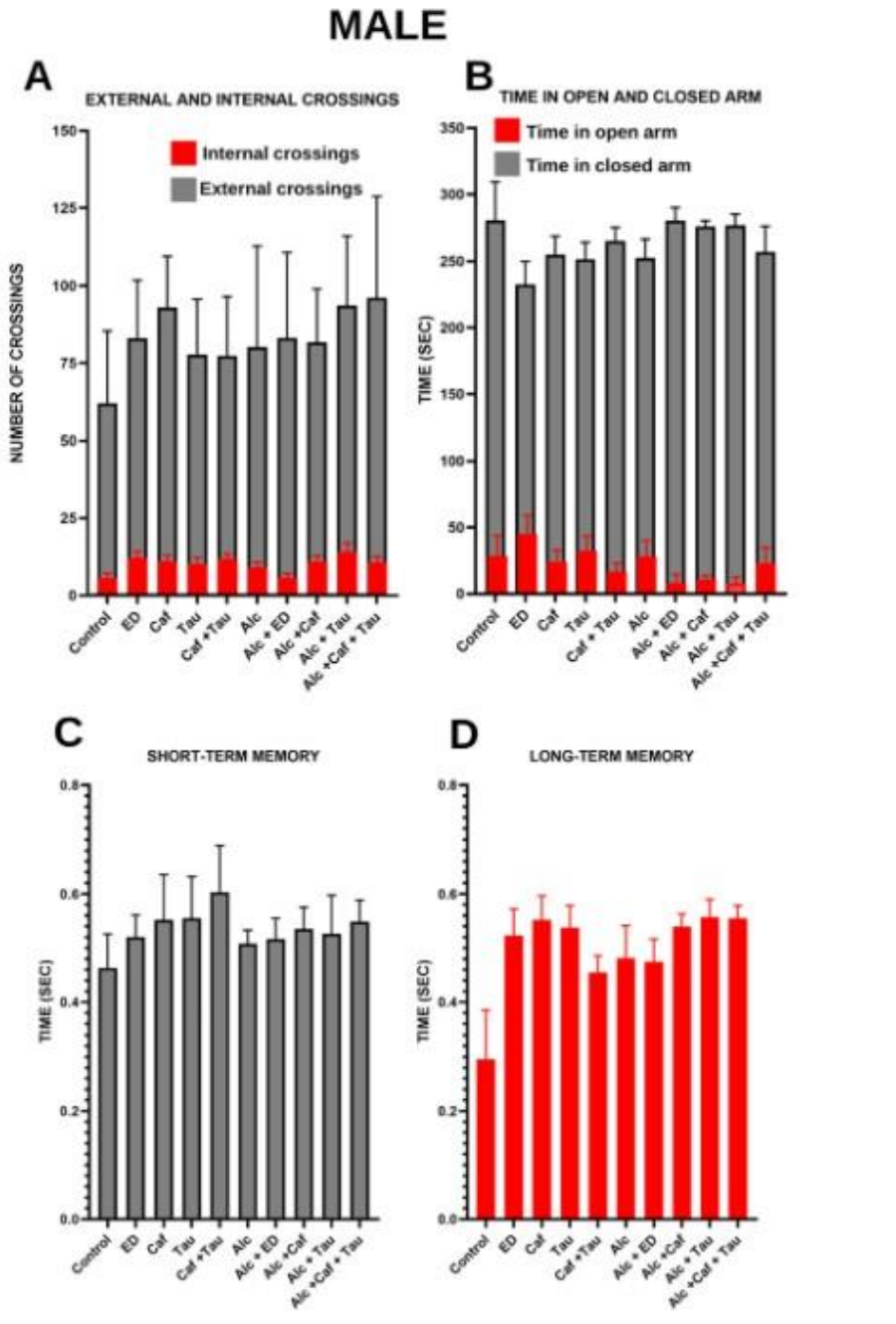

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Fig. 3. Effects of alcohol, energy drinks and their constituents (caffeine and taurine), isolated or combined, in the behavioral analysis of the binge drinking protocol in male Wistar rats (N = 100). A show the open field test, B shows the plus maze test, and C and D show the object recognition test. Parametric data were assessed by one-way ANOVA (post hoc Bonferroni) ($p = 0.006$), and the results are expressed as the mean ± SEM. The asterisk (*) represents a difference between the groups in relation to the control group. Abbreviations: ED—energy drink; Caf—caffeine; Tau—taurine and Alc—alcohol.

of a mixture containing ethyl acetate and hexane (40:60). The samples were then centrifuged for 6 min at 9000 *g*, and the supernatant was withdrawn and dried at 40 °C under a nitrogen stream. After cooling, the residue was reconstituted in 50 μL of acetonitrile fortified with 0.1 % formic acid and transferred to an autosampler vial, and an aliquot of 3 μL was injected into the analytical system.

A Nexera UFLC system coupled to a LCMS-8040 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) was used for the analysis. The ESI-MS/MS parameters were set as follows: 4500 V; desolvation line temperature, 250 °C; heating block temperature, 400 °C; drying gas, 15 L/min; and nebulizing gas, 2 L/min. Collision-induced dissociation was obtained with 230 kPa argon pressure. Analyses were carried out with multiple reaction monitoring (MRM) by using the following fragmentations: m/z 289.20 → m/z 109.10; m/z 289.20 → m/z 97.10 and m/z 289.20 → m/z 43.15 for testosterone ($[M + H]^+$). The chromatographic separation was conducted with a Shim-pack column (25 × 4.6 mm, 5 μm particle size) (Shimadzu, Kyoto, Japan) eluted with a flow rate of 0.4 mL/min. The gradient mobile phase system consisted of water (solvent A) and acetonitrile (solvent B), both fortified with 0.1 % formic acid as follows: 0–3 min, 15–100 % of B; 3–3.25 min, 100 % of B; 3.25–3.30 min, 100–15 % of B; 3.30–4.60 min, 15 % of B. The column oven was kept at 25 °C. The data were processed using LabSolutions software (Shimadzu).

2.7. Statistical analysis

Parametric data with a normal distribution analyzed using the Kolmogorov–Smirnov test are presented as the mean ± SEM, and statistical analysis was performed by one-way ANOVA. Qualitative and/or non-normally distributed data are presented as medians and quartiles and were evaluated by the Kruskal–Wallis test. The proportion of oocytes in each degree of degeneration was evaluated by the chi-square test for homogeneity. All analyses were performed using IBM SPSS Statistics version 23.

3. Results

3.1. Female behavioral results

3.1.1. Open field test

Energy drinks, caffeine, taurine, and alcohol treatment in a binge drinking protocol showed that external crossings differed between the control group and the caffeine, alcohol + energy drinks, alcohol + taurine, and alcohol + caffeine + taurine groups ($p = 0.02$, one-way ANOVA) (Fig. 2A). However, the parameters of internal crossings ($p = 0.43$, one-way ANOVA), grooming ($p = 0.81$, Kruskal–Wallis), rearing ($p = 0.66$, Kruskal–Wallis), and latency to leave the first quadrant ($p = 0.20$, Kruskal–Wallis) showed no significant differences.

3.1.2. Plus maze test

The administration of energy drinks, caffeine, taurine, and alcohol in a binge drinking protocol showed that the number of entries into the open ($p = 0.62$, Kruskal–Wallis) and closed arms ($p = 0.068$, Kruskal–Wallis), the length of stay in the open ($p = 0.77$, one-way ANOVA) and closed arms (p = 0.77, one-way ANOVA), and head dipping ($p = 0.30$ Kruskal–Wallis) showed no differences in any of the evaluated parameters (Fig. 2B).

3.1.3. Object recognition

Energy drinks, caffeine, taurine, and alcohol treatment in a binge drinking protocol revealed that short-term memory ($p = 0.12$, one-way ANOVA) and long-term memory ($p = 0.9$, one-way ANOVA) showed no differences between the groups in the time evaluated (Fig. 2C and D).

Regarding the results of the female behavioral analyses, we concluded that the combination of caffeine and taurine improved locomotor activity; furthermore, caffeine alone increased locomotion. Nonetheless, when this association occurred in combination with alcohol, this effect was reduced. Groups treated with taurine and alcohol did not show decreased locomotor activity — taurine was probably responsible for this effect.

3.2. Male behavioral results

3.2.1. Open field test

The administration of energy drinks, caffeine, taurine, and alcohol in a binge drinking protocol demonstrated that external crossings ($p = 0.08$, one-way ANOVA), internal crossings ($p = 0.06$, one-way ANOVA), rearing ($p = 0.22$, one-way ANOVA), and latency to leave the first quadrant ($p = 0.3$, Kruskal–Wallis) showed no significant differences between groups. (Fig. 3A). The only parameter that showed a difference was grooming ($p = 0.01$, Kruskal–Wallis), between the control and alcohol groups.

3.2.2. Plus maze test

Energy drinks, caffeine, taurine, and alcohol treatment in a binge drinking protocol revealed that the number of entries into the open ($p = 0.29$, Kruskal–Wallis) and closed arms ($p = 0.11$, Kruskal–Wallis), the length of stay in the open ($p = 0.21$, one-way ANOVA) and closed arms ($p = 0.39$, one-way ANOVA), and head dipping ($p = 0.12$, Kruskal–Wallis) showed no differences in any of the parameters evaluated (Fig. 3B).

3.2.3. Object recognition

The administration of energy drinks, caffeine, taurine, and alcohol in a binge drinking protocol resulted in differences in long-term memory ($p = 0.006$, one-way ANOVA) between the control group and the caffeine, taurine, alcohol + caffeine, alcohol + taurine and alcohol + caffeine + taurine groups (Fig. 3 D), although there were no differences in short-term memory ($p = 0.95$, one-way ANOVA) (Fig. 3C) between the groups.

Regarding the results of the behavioral analysis in males, we concluded that the effects on memory were improved when caffeine and taurine were administered alone or in combination. Alcohol alone diminished the effects on memory. Nevertheless, when caffeine and taurine were administered in combination with alcohol, this effect did not appear, and long-term memory was maintained.

3.3. Female reproductive parameters

The administration of energy drinks – whose main constituents are caffeine and taurine – and alcohol in a binge drinking protocol was evaluated in terms of female reproductive parameters. Regarding estrous cycle regularity, Table 1 shows the median of the number of estrus events evidenced during a 15-day period, in which no significant differences were observed ($p = 0.217$; Kruskal–Wallis) between the groups or in comparison to the control group, in addition to the median ($p = 0.180$; Kruskal–Wallis) number of days in which females manifested the estrogenic phase (proestrus and estrus) during that time period. The relative mass of female ovaries presented no statistically significant differences between groups when compared to the control group, as

Table 1
Female reproductive parameters of rats treated with a binge drinking protocol of alcohol, energy drinks, and their main constituents, caffeine and taurine.

Reproductive parameters	Control	Energy drink	Caffeine	Taurine	Caffeine + taurine	Alcohol	Alcohol + energy drink	Alcohol + caffeine	Alcohol + taurine	Alcohol + caffeine + taurine	*p* value (statistical test)
Right and left ovaries (relative mass)	0.4120 ± 0.1315	0.4099 ± 0.0373	0.4616 ± 0.0967	0.5135 ± 0.1865	0.4946 ± 0.1393	0.4813 ± 0.1437	0.4664 ± 0.1168	0.4115 ± 0.1245	0.5517 ± 0.1578	0.4812 ± 0.1422	$p = 0.77$ (one-way ANOVA)
Number of estrus events	3 (3:3)	3 (3:3)	3 (3:3)	3 (3:3)	3 (3:3)	3 (3:3)	3 (3:3)	3 (2:3)	4 (3:3)	3 (3:3)	$p = 0.21$ (Kruskal–Wallis)
Estrus + proestrus (days in the estrogenic phase)	6 (6:6)	5 (5:6)	6 (6:9)	6 (6:6)	7 (7:7)	7 (7:7)	7 (6:7)	7 (6:7)	8 (6:8)	7 (6:7)	$p = 0.18$ (Kruskal–Wallis)
Total number of oocytes	10	16	14	21	8	11	7	7	6	10	
Number of oocytes per animal	2 (2:2)	1 (1:6)	2 (2:3)	5 (2:6)	2 (0:3)	2 (2:3)	1 (1:2)	1 (1:2)	2 (0:2)	2 (1:3)	$p = 0.60$ (Kruskal–Wallis)
Grade I %	30	18.75	28.57	23.8	25	36.36	28.57	14.28	0	40	
Grade II %	50	31.25	28.57	38.09	62.5	27.27	42.85	42.85	33.33	30	
Grade III %	10	31.25	35.71	14.28	12.5	27.27	14.28	14.28	33.33	20	$p = 0.61$ (chi-square homogeneity test)
Grade IV %	10	18.75	7.14	23.8	0	9.09	14.28	28.57	33.33	10	
Low degree of degeneration (sum of grades I and II)	80	50	57.15	61.92	87.5	63.64	71.44	57.15	33.34	70	
High degree of degeneration (sum of grades III and IV)	20	50	42.85	38.08	12.5	36.36	28.56	42.85	66.66	30	
Ratio (high/low)	4	1	1.3	1.6	7	1.8	2.5	1.3	0.5	2.3	

Data were analyzed by ANOVA followed by post hoc comparisons (Bonferroni test). Values are expressed as the mean ± SD. For nonparametric data, the results are presented as medians and quartiles and were analyzed by the Kruskal–Wallis test (N = 50).

shown in Table 1 ($p = 0.77$; one-way ANOVA). The oocyte count per animal ($p = 0.60$ Kruskal–Wallis) and oocyte morphology ($p = 0.61$ chi-square homogeneity test) (Table 1) showed no significant differences in any comparison.

3.4. Male reproductive parameters

The relative mass of male sexual organs treated with energy drinks and their main constituents, caffeine and taurine, combined with alcohol in a binge drinking protocol displayed no significant difference between the groups. Similarly, no significant difference was demonstrated in comparison to the control group, as shown in Table 2 (testicle (right and left) $p = 0.82$ one-way ANOVA; epididymis (right and left) $p = 0.85$ Kruskal–Wallis; prostate $p = 0.87$ Kruskal–Wallis; seminal vesicle $p = 0.95$ Kruskal–Wallis). Likewise, there were no statistically significant differences in cell counts of spermatids ($p = 0.47$, Kruskal–Wallis) collected from the testicle or in sperm obtained from the epididymis ($p = 0.37$, one-way ANOVA) and counted in the Neubauer chamber, as presented in Table 2.

In the estimated morphological evaluation of sperm, tail and head defects showed no differences among the groups ($p = 0.15$, one-way ANOVA) (Table 2). The pathologies of folded tail ($p = 0.32$, Kruskal–Wallis), malformed head ($p = 0.14$, Kruskal–Wallis), short tail ($p = 0.79$, Kruskal–Wallis), curled tail ($p = 0.51$, Kruskal–Wallis), small head ($p = 0.51$, Kruskal–Wallis), and detached head ($p = 1.00$, Kruskal–Wallis) showed no differences (Table 2). Furthermore, there were no significant differences in regard to the serum testosterone level of the treated animals ($p = 0.84$, Kruskal–Wallis) (Table 2).

4. Discussion

The administration of energy drinks, caffeine, taurine, and alcohol in a binge drinking protocol demonstrated no signs of toxicity. The female animals in the caffeine and taurine groups showed increased locomotor activity in the external crossing parameter in the open field test. The plus maze test is commonly used to analyze the levels of anxiety, and the treatments (energy drinks, caffeine, taurine, and alcohol) in our study (binge drinking protocol) showed that there were no effects in female or male rats. Regarding the effects on memory, we selected the object recognition test. In males, treatment with caffeine and taurine alone or in combination improved long-term memory.

In this study, it is paramount to point out that the evaluations were performed during a time period without administrations, being an intermittent protocol of repeated exposure, since the study aimed to assess the long-term effect of exposure, and in a similar way, on how alcohol is usually consumed by adolescents (binge drinking).

It is important to emphasize that in most behavioral studies, the tests are only performed on males. This brings innovation to our study because we analyzed the behavioral and reproductive parameters of both females and males. However, males and females have differences in relation to drug use and abuse. Some evidence suggests that females are more vulnerable than males to the effects of drug abuse during the acquisition, maintenance, and relapse phases (Lynch, 2006). Conversely, males are more sensitive than females to the aversive effects of drugs, such as drug withdrawal (Carroll and Anker, 2010). In our study, females had an improvement in locomotor activity, whereas males had an improvement in memory, mainly in the animals treated with caffeine and taurine. These results indicate that female and male rats showed differences in behavior.

The open field test demonstrated differences in the number of external crossings in females treated with caffeine, alcohol + energy drinks, alcohol + taurine, and alcohol + caffeine + taurine. In a previous study, it was found that in Swiss mice (40 days) treated acutely with alcohol (4 g/kg 40 %) and energy drinks (8 mL/kg), the combination of energy drinks and alcohol increased locomotor activity (Krahe, 2017). Similar to our results, the energy drinks were repeatedly responsible for the improvement in locomotor activity, possibly due to the constituents of energy drinks (caffeine and taurine) performing a stimulating function. According to Cappelletti et al. (2015), caffeine is related to an enhancement in physical performance, in addition to being a stimulant

Table 2
Male reproductive parameters of rats treated with the binge eating protocol of alcohol, energy drinks, and their main constituents, caffeine and taurine. Data were analyzed by ANOVA followed by post hoc comparisons (Bonferroni test). Values are expressed as the mean ± SD. For nonparametric data, the results are presented as medians and quartiles and were analyzed by the Kruskal–Wallis test ($N = 50$).

Reproductive parameters	Control	Energy drink	Caffeine	Taurine	Caffeine + taurine	Alcohol	Alcohol + energy drink	Alcohol + caffeine	Alcohol + taurine	Alcohol + caffeine + taurine	p value (statistical test)
Right and left testicles (relative mass)	0.91 ± 0.08	1.03 ± 0.18	1.00 ± 0.19	0.98 ± 0.19	0.78 ± 0.45	0.98 ± 0.21	0.91 ± 0.09	0.89 ± 0.08	0.92 ± 0.12	0.94 ± 0.11	$p = 0.82$ (one-way ANOVA)
Right and left epididymis (relative mass)	0.14 (0.14; 0.18)	0.17 (0.16; 0.17)	0.18 (0.16; 0.18)	0.15 (0.15; 0.20)	0.15 (0.15; 0.16)	0.17 (0.14; 0.18)	0.15 (0.15; 0.16)	0.15 (0.14; 0.15)	0.16 (0.14; 0.16)	0.17 (0.14; 0.17)	$p = 0.85$ (Kruskal–Wallis)
Prostate (relative mass)	0.04 (0.04; 0.05)	0.05 (0.05; 0.08)	0.05 (0.05; 0.06)	0.05 (0.04; 0.06)	0.05 (0.04; 0.07)	0.06 (0.04; 0.07)	0.04 (0.04; 0.06)	0.04 (0.04; 0.06)	0.04 (0.04; 0.05)	0.04 (0.04; 0.06)	$p = 0.87$ (Kruskal–Wallis)
Seminal vesicle (relative mass)	0.07 (0.07; 0.09)	0.08 (0.07; 0.08)	0.08 (0.07; 0.09)	0.06 (0.05; 0.11)	0.08 (0.07; 0.09)	0.07 (0.06; 0.08)	0.07 (0.07; 0.10)	0.08 (0.06; 0.09)	0.07 (0.07; 0.07)	0.07 (0.07; 0.07)	$p = 0.95$ (Kruskal–Wallis)
Spermatids (testicle)	64.25 (51.5; 71.25)	37.75 (36.5; 44.25)	46.25 (26.25; 49.25)	42 (41.5; 50.5)	34.5 (12.5; 46.5)	47.5 (39.25; 52.5)	46.25 (45.5; 49.75)	49.5 (11; 54)	45.5 (40; 63.75)	49.25 (47; 51.25)	$p = 0.47$ (Kruskal–Wallis)
Sperm (right and left epididymis)	42.8 ± 21.70	43.3 ± 27.62	35.65 ± 8.53	34.55 ± 14.63	20.45 ± 17.52	49.15 ± 29.68	51.05 ± 21.44	48.2 ± 24.59	48.35 ± 23.26	58.25 ± 27.75	$p = 0.37$ (one-way ANOVA)
Sperm morphological defects (head and tail)	25.6 ± 3.13	27.25 ± 3.77	33.8 ± 2.77	32.2 ± 14.07	40.75 ± 11.5	37.6 ± 4.97	33.6 ± 3.64	32.2 ± 4.20	35 ± 10	35.8 ± 8.43	$p = 0.15$ (one-way ANOVA)
Testosterone level	2.02 (1.31; 2.72)	3.35 (1.8; 4.56)	2.38 (1.4; 2.84)	2.18 (1.62; 3.25)	3.77 (1.61; 4.7)	3.23 (1.33; 5.2)	2.16 (1.74; 3.31)	2.15 (1.53; 2.77)	3.13 (1.5; 8.95)	3.5 (1.87; 4.62)	$p = 0.84$ (Kruskal–Wallis)

of locomotor activity at lower doses. In a study conducted by Heidari et al. (2018), taurine (50, 100 and 200 mg/kg, gavage) administered for 28 days to animals with cirrhosis increased locomotor activity through the open field test.

The effect of alcohol consumption (3.4 g/kg) with or without energy drinks (10.71 mL/kg) for 6 consecutive days was also evaluated in behavioral tests (locomotor activity test, object recognition test, test of social discernment, and place preference conditioning test) in male Wistar rats by Takahashi et al. (2015). The acute binge-like ingestion of alcohol decreased locomotor activity and caused deficits in both the object recognition and social discrimination tests. In addition, adding the energy drink to the alcohol solution did not modify these effects. However, our results showed that the main components of energy drinks (caffeine and taurine) increased locomotion in females, even when the evaluation occurred a few days after the last administration.

Regarding the object recognition test in males, the long-term memory results showed an increased recognition index in the groups treated with caffeine and taurine, even in those treated with the combination of alcohol. Some of the effects of taurine are attributed to a role in memory function through modulation of *N*-methyl-D-aspartate (NMDA) receptors. However, no human studies have established a concrete relationship between taurine and memory improvement (Bichler et al., 2006). Additionally, according to Valle et al. (2018), the administration of caffeine (3.2 mg/kg) and taurine (40 mg/kg) for a period of 28 days in adult male Wistar rats improved memory performance in the object recognition test. In the aforementioned study, the administered doses were the same as those used in this work, but the administration time was longer (28 days) and in a different period (in adult rats). It is noteworthy in this work that even in a shorter period of administration, a higher recognition index was obtained.

The ovary mass and the estrous cycle of prepubertal females did not present any sign of alterations. Similar to females, males substantiated no significant differences in the parameters evaluated. The relative mass of sex organs and the production of spermatids and sperm were similar between the groups. The analysis of the estrous cycle, including the number of days the animals were in the estrogenic phase and the number of estrus events, showed no change in our evaluations. According to Ghosh and Maiti (2015), taurine alone is not capable of causing changes in ovary mass or in the estrous cycle. In his study with 36 female Wistar rats, the animals that received 50 mg/kg of taurine for 14 consecutive days maintained ovary mass and estrous cycle regularly in both the estrogenic and the estrous phases. When analyzing the results obtained in the present study regarding the degree of maturation of oocytes, an improved result was also observed in the group of females treated with caffeine + taurine, which corroborates the hypothesis of Xia et al. (2017). The aforementioned authors cultured mature oocytes from aged mice in vitro at a concentration of 5 mM caffeine for 24 h; therefore, their studies suggested that caffeine could inhibit oocyte aging in mice and maintain cell viability.

Concerning the reproductive analysis performed on females, no damage was generated, but the count of oocytes per group indicated a possible decrease in the groups treated with alcohol. The same variation occurred in the degrees of degeneration of oocytes, in which a higher percentage of degrees III and IV (indicating more advanced degeneration) was observed in females treated with energy drinks (higher ratio between high/low). In contrast to energy drinks, the combination of caffeine and taurine in our study showed better results in terms of oocyte quality (higher frequency of grades I and II). These findings support the hypothesis that taurine plays a protective role in reproduction and acts as a support for improved fertilization (Mu et al., 2015).

Studies evaluating the toxic potential of alcohol on female reproduction suggest that excessive drinking exerts marked reproductive toxicity, such as decreased fertility, ovulation obstruction, and reduced ovary mass (Liu et al., 2018). However, despite data from the literature reinforcing that alcohol can be potentially toxic, it was not possible to draw significant findings to support these hypotheses. Cebral et al. (2011) demonstrated pertinent changes regarding the toxicity that alcohol has in relation to reproductive parameters. In their study, after 35 superovulated female mice (60 days) were treated with alcohol (10 g/kg) for 27 days and euthanized in the estrogenic phase, it was possible to visualize nuclear anomalies in the oocytes, such as significantly smaller amounts of cells and more degrees of severe degeneration (grades III and IV). This represented approximately 55.5 % of the oocytes analyzed when compared to the control group (water). In our

study, prepubertal females were not superovulated, and the concentration of alcohol received was lower (2 g/kg) in an intermittent model. Euthanasia did not always occur during the estrogenic phase, showing no alterations in these conditions.

The relative mass of male reproductive organs in our study did not change for any of the compounds. However, according to Reddy and Reddy (2015), adult Wistar rats (90 days) treated with 2 g/kg alcohol for 60 days showed important decreases in the relative mass of the testicles, epididymis, seminal vesicle, and prostate. In contrast, the treatment time in our study was 28 days, approximately half the administration period used by the aforementioned authors (60 days), and the age of the animals was different between the studies. Furthermore, our administrations were carried out only 3 times a week, which may have contributed to the lack of alterations. Although the deleterious effects of alcohol on sperm count are consistent in the literature, no changes were observed in our study. According to Dhawan and Sharma (2002), male Wistar rats that received 3 g/kg of alcohol for 30 days had reduced sperm concentrations. In disagreement with that, the animals in our study were treated with 2 g/kg alcohol in a binge drinking model that differed in dose, frequency, and duration.

According to Amann (1986), the main variables of male reproductive toxicity are the relative mass of the testicles, testicular morphology, number of sperm and sperm morphology. The relative mass of sex organs, as well as sperm and sperm cell counts, were not altered in this study. Despite the profile of the main consumers of energy drinks being young males of childbearing age, previous findings on the effects of energy drinks on reproductive parameters are not described in the literature (Mansour et al., 2019). In our study, no deleterious effects were found on reproductive parameters in the groups that received energy drinks combined with alcohol.

5. Conclusion

The results demonstrate that the combination of energy drinks (and their main constituents, caffeine and taurine) and alcohol in a binge drinking model did not cause important alterations in the reproductive profile of male or female prepubertal Wistar rats. Locomotion was increased in females treated with the main constituents of energy drinks (caffeine and taurine), reinforcing the stimulant action of the components. In males, long-term memory also presented better results in these groups, even when combined with alcohol. Future analyses are necessary to understand whether these effects might intensify or appear, especially because of adolescents' chronic consumption of energy drinks and alcohol, whose long-term effects are unknown.

Declaration of competing interest

The authors declare that they have no conflicts of interest to disclose.

Data availability

Data will be made available on request.

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6 CONCLUSÃO

O estudo desenvolvido teve como objetivo avaliar o efeito da associação de energéticos, e seus principais constituintes cafeína e taurina, associados ou não ao álcool através do protocolo *binge drinking* de administração sobre parâmetros hematológicos, bioquímicos, histopatológicos, imunológicos, reprodutivos e comportamentais, em ratos Wistar machos e fêmeas pré-púberes (28-60 dias).

O primeiro artigo elaborado teve como enfoque a avaliação histopatológica, imunológica, bioquímica e hematológica de ratos machos Wistar pré-púberes, através do protocolo *binge drinking* de administração de álcool, energéticos e seus constituintes (cafeína e taurina). Os resultados encontrados demonstraram que a associação de álcool e energéticos, assim como a administração isolada destes componentes, aumentou a produção de citocinas pró-inflamatórias. Mas, quando o álcool foi associado a cafeína e a taurina houve uma diminuição na produção de citocinas. Em relação a avaliação histopatológica foi encontrado dano no fígado e nos rins através da presença de infiltrado celular, vacuolização, degeneração hidrópica, principalmente. Os grupos que apresentaram maior grau de dano foram aqueles tratados com álcool, álcool associado ao energético e álcool associado a cafeína e/ou taurina. Já as avaliações hematológicas e bioquímicas não revelaram alteração em nenhum dos grupos tratados.

O segundo artigo elaborado teve como proposta avaliar o efeito da associação de álcool, energéticos e seus constituintes (cafeína e taurina), através do protocolo *binge drinking* de administração, em ratos Wistar machos e fêmeas sobre efeitos comportamentais e reprodutivos. Os dados reprodutivos encontrados não demonstraram prejuízo em nenhuma das análises realizadas tanto nos machos quanto nas fêmeas. Nos testes comportamentais pode-se observar apenas diferença no teste de locomoção nas fêmeas, havendo um aumento nos cruzamentos externos nos grupos tratados com cafeína, álcool + energético, álcool + taurina e álcool + cafeína + taurina, sendo um possível indicativo de aumento da atividade locomotora gerado pela ingesta destes componentes. Em relação aos machos houve uma melhora na memória de longa duração nos grupos tratados com cafeína, taurina, álcool + cafeína, álcool + taurina e álcool + cafeína + taurina. No entanto, este aumento de retenção de memória de longa duração não foi encontrada nas fêmeas, e estes componentes, cafeína e taurina, são descritos na literatura como intensificadores de memória.

De forma geral, os resultados encontrados em ambos os artigos, sugerem que os constituintes dos energéticos (cafeína e taurina), mesmo administrados em baixas dosagens, e quando associados ao álcool, foram capazes de melhorar os efeitos negativos gerados pela administração do álcool. Além disto, pode-se salientar que o álcool mostrou um potencial tóxico, mesmo após um longo período de sua última administração. O consumo do energético de forma isolada também foi capaz provocar malefícios ao organismo, quando avaliado a produção de citocinas inflamatórias.

O consumo de energéticos associados ao álcool gera grande preocupação por instituições de saúde ao redor do mundo, para a proteção da criança e do adolescente. Portanto, estudos que avaliem seus efeitos é de grande importância. Estudos futuros poderão aumentar os grupos em análise, por exemplo os açúcares presentes nos energéticos, que podem causar alteração comportamental e que mascaram os efeitos da bebida alcoólica aumentando a sua ingesta. Outros protocolos de maior tempo de administração também poderão ser implementados, para avaliar os efeitos a longo prazo.

ANEXO A – PARECER CEUA

CEUA –COMISSÃO DE ÉTICA NO USO DE ANIMAIS

PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA E ENSINO

1) PROTOCOLO Nº: 221/17

2) DATA DO PARECER: 13/12/2017 **Parecer: 543/17**

3) TÍTULO DO PROJETO:

Avaliação da toxicidade subcrônica de bebida energética associada ao álcool em ratos wistar machos e fêmeas durante a puberdade.

4) PESQUISADOR RESPONSÁVEL:

Pedro Romão

5) RESUMO DO PROJETO:

O presente projeto avaliará as bebidas energéticas e seus constituintes as quais são extensamente utilizadas pelos jovens. Tais bebidas são compostas de substâncias estimulantes as quais podem alterar comportamentos em humanos, além de outros parâmetros fisiológicos.

6) OBJETIVOS DO PROJETO:

Avaliar a toxicidade de bebida energética e de seus constituintes como cafeína e taurina isolados e em associação com o álcool em ratos Wistar pré-púberes, por meio de parâmetros comportamentais, imunológicos, bioquímicos, oxidativos e histopatológicos.

7) FINALIDADE DO PROJETO: [ ] Ensino [X] Pesquisa

8) ITENS METODOLÓGICOS E ÉTICOS DO PROJETO:

Título	[X] Adequado	[ ] Comentários
Introdução	[X] Adequada	[ ] Comentários
Objetivos	[X] Adequados	[ ] Comentários
Relevância e Justificativa	[X] Adequados	[ ] Comentários

Materiais e Métodos [X] Adequados [ ] Comentários

Cronograma para execução da pesquisa [X] Adequado [ ] Comentários

Orçamento e fonte financiadora [X] Adequados [ ] Comentários

Referências Bibliográficas [X] Adequadas [ ] Comentários

9) O PROJETO ESTÁ ADEQUADO À LEGISLAÇÃO VIGENTE:

[X] Sim [ ] Não

10) INFORMAÇÕES RELATIVAS AOS ANIMAIS:

Grau de dor/estresse: **B** | **C** [x] **D** [ ] **E** [ ]

Justifique:

Eutanásia realizada sem anestesia. Justificativa: o uso de anestésicos poderia interferir nas análises realizadas no estudo

Espécie: Ratos Wistar **Número Amostral:** 200

Redução Amostral: [ ] Sim [x] Não

Justifique:

Substituição de Metodologia: [ ] Sim [x] Não

Se achar necessário, justifique e sugira uma nova metodologia:

Aprimoramento da Metodologia: [ ] Sim | **x** | Não

Se achar necessário, justifique e sugira aprimoramentos da metodologia:

Acomodação e manutenção dos animais: [x] Adequada | | Inadequada

Se achar inadequada cite abaixo as melhorias necessárias:

Manipulação dos animais: [x] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias:

Analgesia dos animais (se aplicável): [ ] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias com analgésico substituto:

Não se aplica

Anestesia dos animais (se aplicável): [ ] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias com anestésico substituto:

Não se aplica

Eutanásia dos animais (se aplicável): [X] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias com metodologia substituta:

Local de Realização (Biotério/Labotarório): Laboratório de Imunologia Celular e Molecular

Outra instituição. Qual?

11) CRONOGRAMA DE UTILIZAÇÃO DE ANIMAIS

Data	Espécie	Sexo	Quantidade
2018	**Ratos wistar**	**Machos e fêmeas**	**100 machos e 100 fêmeas**

12) RECOMENDAÇÃO: As pendências deverão ser respondidas em uma carta, indicando as páginas do projeto que foram alteradas (nova versão), assinadas pelo pesquisador responsável.

[X] Aprovado

[ ] Com Pendência

[ ] Não aprovado

Data de início/10/2017 Data de Término/10/2020

Comentários gerais sobre o projeto:

Os questionamentos foram respondidos de maneira adequada.

ANEXO B – Normas de formatação para as revistas científicas

Seguindo as normas da coordenação do PPG-Ciências da Saúde, optou-se por inserir apenas o link para as normas online de cada revista.

Artigo 1

Revista Toxicology and Applied Pharmacology (*IF*: 4.21).

https://www.elsevier.com/journals/toxicology-and-applied-pharmacology/0041-008X/guide-for-authors#

Artigo 2

Revista Pharmacology, Biochemistry and Behavior *(IF:3.67)*

https://www.elsevier.com/journals/pharmacology-biochemistry-and-behavior/0091-3057/guide-for-authors